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The MAR1 Transporter of *Arabidopsis thaliana* Has Roles in Aminoglycoside Antibiotic Transport and Iron Homeostasis

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The MAR1 Transporter of *Arabidopsis thaliana* Has Roles in Aminoglycoside Antibiotic Transport and Iron Homeostasis

by

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Dedication

To my father, John Conte, my brother, Aaron Conte, and the memory of my mother,

Michelle Anne Burke Conte

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The MAR1 Transporter of *Arabidopsis thaliana* Has Roles in Aminoglycoside Antibiotic Transport and Iron Homeostasis

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Widespread antibiotic resistance is a major public health concern, and plants represent an emerging antibiotic exposure route. Recent studies indicate that crop plants fertilized with antibiotic-laden animal manure accumulate antibiotics, however, the molecular mechanisms of antibiotic entry and subcellular partitioning within plant cells remain unknown. Here we report that mutations in the *Arabidopsis* locus *Multiple Antibiotic Resistance* (*MAR1*) confer resistance, while *MAR1* overexpression causes hypersensitivity to multiple aminoglycoside antibiotics. Resistance is highly specific for aminoglycosides and does not extend to antibiotics of other classes, including the aminocyclitol, spectinomycin. Yeast expressing *MAR1* are hypersensitive to the aminoglycoside, G418, but not to chloramphenicol or

cycloheximide. *MAR1* encodes a protein with 11 putative transmembrane domains with low similarity to ferroportin1 from *Danio rerio*. A MAR1:YFP fusion protein localizes to the chloroplast, and chloroplasts from plants overexpressing MAR1 accumulate more of the aminoglycoside, gentamicin, while *mar1-1* mutant chloroplasts accumulate less than wild type. *MAR1* overexpression lines are slightly chlorotic, and this chlorosis is rescued by application of exogenous iron. *MAR1* expression is also downregulated by low iron. Taken together, these data suggest that MAR1 is a plastid transporter that is likely to be involved in cellular iron homeostasis, and allows opportunistic entry of multiple antibiotics into the chloroplast.

mar1 mutants represent an interesting example of plant antibiotic resistance that is based on the restriction of antibiotic entry into a subcellular compartment. Knowledge about this process – and other processes of antibiotic entry – could enable the production of crop plants that are incapable of antibiotic accumulation, aid in development of phytoremediation strategies for decontamination of water and soils polluted with antibiotics, and further the development of new plant-based molecular markers. The work described here also contributes to our understanding of how plants interact with the antibiotics they encounter, both in the laboratory (where aminoglycosides such as kanamycin are used heavily to select for transgenics) and in the natural environment.

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CHAPTER I

Introduction: Antibiotics and Plants

Antibiotic use in Agriculture

Antibiotics play an essential role in the treatment of bacterial infections. However, whenever bacteria are exposed to antibiotics, resistant strains are selected for. Thus, while antibiotics are important for disease treatment, their use tends to create strains that are more antibiotic resistant over time. For this reason, it is recommended that antibiotics be used only when absolutely necessary. Unfortunately, overuse of antibiotics is common in both medicine and agriculture.

The amount of antibiotics used non-therapeutically in agriculture is estimated to be eight times greater than the amount used in all of human medicine (Mellon et al., 2001), and this use accounts for about 70% of total antibiotic use in the United States (Florini et al., 2005). Many of these antibiotics are not well absorbed in the animal gut and are excreted largely unchanged in manure (Mackie et al., 2006; Sarmah et al., 2006). In fact, as much as 90% of certain antibiotics can be excreted as the parent compound (Kumar et al., 2005). Antibiotic concentrations in manure can vary, but typical concentrations tend to be in the 1 to 10 mg kg^{-1} or L^{-1} range (Kumar et al., 2005). When manure is spread as fertilizer, these antibiotics contaminate the environment, and many can retain antimicrobial activity in soil for long periods of time (Chander et al., 2005).

Land application of manure is a common practice for supplying nutrients to crops. The use of antibiotic-laced manure as fertilizer appears to be the dominating pathway for the release of antibiotics into the environment (Baguer et al., 2000). A growing body of research shows that land-applied antibiotics in manure contaminate surface and ground waters (Kay et al., 2004; Burkhardt et al., 2005; Kay et al., 2005; Kreuzig et al., 2005; Davis et al., 2006), and that these antibiotics are contributing to the development and spread of resistance (Khachatourians, 1998; Phillips et al., 2004; Pruden et al., 2006). Considering that the U.S. livestock population is four times larger than the U.S. human population, and the total quantity of manure produced by these animals is roughly 132 million metric tons (Dolliver et al., 2007), the presence and persistence of antibiotics in such a large quantity of manure represents a significant environmental problem.

Manure is typically land-applied either as raw manure (fresh or dried) or as compost. Composting is an effective way of reducing antibiotic contamination before land application (Dolliver et al., 2008). However, not all manure is composted, and composting is known to concentrate heavy metals, thus making the compost potentially more hazardous than the manure it was created from (Kuepper, 2003). Therefore, farmers must weigh the pluses and minuses of using compost versus raw manure to fertilize their crops. In organic farming, use of composted manure is recommended, and the use of raw manure is tightly regulated due to concerns over bacterial (*Salmonella* and *E. coli*) contamination (Kumar et al., 2005). Regulations for certified organic farmers state that raw manure must be applied no less than 90-120 days before harvest, depending on the crop (Guan and Holley, 2003). However, antibiotics are known to persist in soil

for much longer. For example, virginiamycin has a half-life of 87 to 173 days in sandy silt and silty sand soils (Weerasinghe and Towner, 1997), sarafloxacin (an antibiotic used widely in poultry production) was found to be less than 1% degraded after 80 days in soil (Marengo et al., 1997), and apramycin (an aminoglycoside often given to swine) retains 75% of its activity after two years in soil (Environmental Assessment for Apralan Premix for Swine, 1985). Despite this known persistence, there are still no guidelines for the presence of antibiotics in manure (Kumar et al., 2005).

Until recently, major concerns about antibiotic use in agriculture have been related to their presence in animal-based food products (such as milk and meat) and their contamination of the water supply via farm runoff. However, crop plants fertilized with antibiotic-contaminated manure are now emerging as an additional antibiotic exposure route. Several recent studies have shown that various crops can accumulate measurable levels of antibiotic after growth on contaminated soils (Migliore et al., 2003; Kumar et al., 2005; Boxall et al., 2006; Dolliver et al., 2007). It is well known that entry of antibiotics into bacterial cells is mediated by active transport systems (Chopra, 1988; Scholar and Pratt, 2000), and there is evidence now that uptake of antibiotic into plants is also an energy-dependent process (Kong et al., 2007). Unfortunately, the specific plant transporter proteins capable of recognizing and moving antibiotics remain unknown.

Antibiotics in the Natural Environment

Bacteria are the most numerous soil-dwelling organism – every gram of soil contains millions of bacteria (Sullivan 2004). One very common group of soil dwelling higher

bacteria, the actinomycetes, are major antibiotic producers (D'Costa et al., 2006). The actinomycete genus *Streptomyces* synthesizes over half of all known antibiotics, including the aminoglycosides kanamycin, streptomycin, and apramycin (Crandall and Hamill, 1986). Some types of actinomycetes are nitrogen-fixers and can form mutualistic associations with plants – the plant provides valuable nutrients while the bacterium makes reduced nitrogen available to the plant (Valdes et al., 2005). Others are endophytes – *Streptomyces* strains isolated from tomato roots were shown to have both antibacterial and antifungal properties, thus promoting growth and enhancing disease resistance in these plants (Cao et al., 2004). Interestingly, many agricultural products sold to prevent plant diseases are actinomycete spore cultures (for example *Streptomyces griseoviridis* sold as Mycostop by Kemira Agro in Finland).

The antimicrobials produced by plant-associated actinomycetes have the potential to be both beneficial and harmful to the plants they colonize. It was demonstrated that, while many *Streptomyces* strains had beneficial effects in alfalfa, several strains caused a marked reduction in plant dry weight (Samac et al., 2003). Whether beneficial or not, it seems safe to assume that plants have endured antimicrobial exposure – either from their own endosymbionts or other soil dwelling bacteria – as long as the two have coexisted. Still, unanswered questions remain about the molecular nature of this interaction. Specifically, how do antimicrobials move within the plant body? How do they move within the plant cell? How do they access their targets? The research described in this thesis aims to begin answering these questions.

Antibiotics and Plants

It is widely recognized that plants are sensitive to many antibiotics, and this fact has been exploited for the benefit of both basic and applied plant science to produce transgenic plants. Transgenic plant selection systems are typically based on bacterial genes that provide resistance to antimicrobial compounds. These include: kanamycin resistance conferred by a phosphotransferase (Fraley et al., 1983); gentamicin resistance conferred by acetyltransferases (Hayford et al., 1988); spectinomycin and streptomycin resistance conferred by adenyltransferases (Svab and Maliga, 1993); hygromycin resistance conferred by a phosphotransferase (Lloyd et al., 1986); and bialophos resistance (the herbicide BASTA is a derivative called phosphinothricin) conferred by an acetyltransferase (De Block et al., 1987). It is interesting to note that these are all modification enzymes specific for one or a few particular antibiotics, mostly aminoglycosides.

Generally, the aminoglycoside antibiotics target the translational machinery of prokaryotes. Because the eukaryotic organellar translational machinery is prokaryotic in nature, these antibiotics target chloroplast and mitochondrial translation in plants. Thus, endogenous, high-level resistance to aminoglycosides in plant lines is typically due to specific changes in organellar ribosomal subunits. These include mutations in the *Nicotiana tabacum* chloroplast 16S rRNA that probably affect the tRNA binding and confer resistance to spectinomycin (Fromm et al., 1987; Svab and Maliga, 1993), mutations in the *Solanum nigrum* chloroplast 16S, 23S and rps12(3') ribosomal genes that confer resistances to spectinomycin, streptomycin and lincomycin (Kavanagh et al.,

1994), and a mutation in the alfalfa 16S plastid rRNA that confers kanamycin resistance (Rosellini et al., 2004). The 16S rRNA is part of the 30S ribosomal subunit, and these plant mutations generally reflect the same kinds of target site mutations that can be found in prokaryotes. In the few cases where they were analyzed, it was found that these mutations were strictly maternally inherited, as would be expected for organellar genomes.

Plants are not generally sensitive to antibiotics that target prokaryotic pathways not present in plant cells. For example, the beta-lactams target the bacterial cell wall by inhibiting transpeptidation via covalent linkage to a transpeptidase, thus preventing the crosslinking of new wall peptides. Since plant and bacterial cell walls are distinct, plants are generally immune to high concentrations of these antibiotics. In fact, the beta lactams are often used in plant cell cultures to help keep them aseptic.

Unlike in plant systems, antibiotic resistance in bacterial systems is a heavily studied area and it cannot be thoroughly reviewed here. Modes of antibiotic resistance include (but are not limited to) enzymatic inactivation of the antibiotic (such as phosphorylation of neomycin), degradation of the antibiotic, alteration of the antibiotic target such that the antibiotic can no longer bind (such as changes in ribosomal structure), reduced uptake of the antibiotic, or increased efflux of the antibiotic via modification of membrane-bound transporters (Davies, 1997). Multiple antibiotic resistance can be mediated in *E. coli* by activation of the *Mar* locus through chemical activators, mutational activation of the marA activator, or knock down of the marR repressor. MarA can modulate the regulation of over 60 genes that have to do with tolerating oxidative

stress, antibiotic challenge and disinfectants (Barbosa and Levy, 2000), and some of these genes encode for efflux pumps.

Multidrug resistance in bacteria is often conferred by expression of multidrug efflux transporters encompassing several protein families including ATP binding cassette (ABC) transporters, the major facilitator superfamily (MFS), the multidrug and toxic compounds efflux (MATE) family and others (Paulsen, 2003). These pumps use ATP hydrolysis, proton gradients, and sodium ion gradients, respectively, as energy sources to drive the efflux. A common feature that allows for efflux of compounds comprising very different structures seems to be the presence of a large cavity that is flexible and hydrophobic. Bacteria – especially soil dwelling and plant-associated bacteria – have large numbers of these efflux pumps in their genomes.

There are only a few reports of antibiotic resistance in plants that are based on overexpression of efflux transporters. In one study, it was shown that overexpression of an endogenous *Arabidopsis thaliana* ABC transporter, Atwbc19, confers kanamycin resistance in plants (Mentewab and Stewart, 2005). Levels of resistance were comparable to levels attained through expression of the bacterial neomycin phosphotransferase II (*nptII*). In this case, resistance was found to be specific for kanamycin – resistance to other aminoglycoside antibiotics was not conferred. It was also shown that a plant mutated for this gene was more sensitive to kanamycin. Protein subcellular localization studies were somewhat inconclusive and more work remains to be done on this particular transporter. However, its mere existence is evidence that there

are probably more genes like it that have yet to be uncovered, and begs the question as to what the normal function of these transporters might be.

Our lab has studied the effect of overexpressing an apyrase gene from pea and the *Arabidopsis Multi Drug Resistance1* (MDR1, an ATP Binding Cassette protein) gene. We found that either could confer resistance to multiple herbicides and a single antibiotic, cycloheximide (Windsor et al., 2003), but not kanamycin or other antibiotics. We also showed that inhibition of ectoapyrase reversed this resistance (Windsor et al., 2002).

Despite the major role of efflux transporters in generating resistance, the importance of influx transporters must not be ignored. Antibiotics obviously must enter the cell in order to function, and a block of entry can also be sufficient to generate resistance. For example, in Gram-negative bacteria, porins in the outer membrane are a major point of entry for antibiotics, and the modification of these porins (such that entry is reduced) can increase resistance to antibiotics (Pages et al., 2008). Perhaps not surprisingly, movement of aminoglycoside antibiotics across the bacterial inner membrane involves energy-dependent transport (Taber et al., 1987; Mao et al., 2001). It is thought that aminoglycosides, due to their structural similarities with polyamines, may utilize polyamine transport systems for entry into bacteria and some eukaryotic cells (Van Bambeke et al., 2000). In fact, links have been established between decreased levels of oligipeptide binding protein (OppA), a component of the oligopeptide transport system, and increased resistance to aminoglycosides in *E. coli* (Kashiwagi et al., 1998; Acosta et al., 2000). However, a recent conflicting report stated that the Opp uptake system does

not play a role in aminoglycoside uptake (Nakamatsu et al., 2007). Thus, the actual import proteins capable of aminoglycoside transport still remain unknown.

Clues about the nature of antibiotic import proteins may also be obtained from knowledge of antibiotic efflux pumps. For example, the cmr chloramphenicol efflux pump bears sequence similarity to several sugar transporters (Nilsen et al., 1996). The *mexAB*/*oprM* multidrug efflux operon of *P. aeruginosa* is involved in efflux of tetracycline, chloramphenicol, several quinolones, and a range of β-lactams (Paulsen et al., 1996). It is also regulated by iron concentration, and proposed to be involved in the secretion of the iron chelator, pyoverdine, under conditions of iron starvation (Poole et al., 1993; Poole et al., 1993). The *B. subtilis* Blt drug transporter exports chloramphenicol and puromycin (Neyfakh et al., 1991) as well as polyamines (Jack et al., 2001). Therefore, like these antibiotic export proteins, antibiotic importers may also be transporters of common metabolites that have been hijacked by antibiotic, thus allowing access to intracellular targets.

The Need for New Plant-Based Resistance Markers

The most common, and perhaps the first marker gene used for generating transgenic plants is yet another aminoglycoside modifying enzyme, neomycin phosphotransferase II, or NPTII, from the *Escherichia coli* transposon, Tn5. NPTII confers resistance to the aminoglycosides neomycin, kanamycin (Fraley et al., 1983), geneticin (G418) (Miki and McHugh, 2004) and paromomycin (Patnaik and Khurana, 2003). In 2002, 70% of transgenic plant studies appearing in *Plant Molecular Biology* utilized the *NPTII* gene as

a selectable marker (Miki and McHugh, 2004). Additionally, *NPTII* is the most widely used marker in transgenic crops, and is found in many of the crops currently approved for commercial production. In 2001-2002, *NPTII* was the most prevalent antibiotic selectable marker used in US field trials (Miki and McHugh, 2004).

Whether a real or perceived problem, public concern over the spread of antibiotic resistance through horizontal gene transfer (of *NPTII* or other bacterial resistance genes) has limited the acceptance of genetically modified plants, especially in Europe (Conner et al., 2003). Steps are currently being taken to reduce risk by phasing out the use of antibiotic resistance markers in plant systems, with the ultimate goal being to eliminate all bacterial antibiotic resistance genes from GM plants as new technologies become available (Joersbo, 2001). Some examples of new technologies include the use of alternate, non-antibiotic-resistance markers (Joersbo et al., 1998), "removable" antibiotic-resistance genes that can be excised via transposons or site-specific recombination prior to distribution of the plant for commercial use (Dale and Ow, 1991; Iamtham and Day, 2000; Zuo, 2001), and genes that will inactivate if transferred to a bacterial cell environment (Libiakova et al., 2001). Despite these efforts, the bacterial resistance gene *NPTII* still remains the most commonly used marker (Libiakova et al., 2001). Clearly, new markers are needed that can alleviate public concern over the use of bacterial resistance genes, while maintaining the simplicity and practicality of *NPTII*.

The discovery and characterization of *AtWBC19* (Mentewab and Stewart, 2005) has sparked the hope that plant-based antibiotic transport proteins may be promising new candidates for selectable markers (Rea, 2005). Since *AtWBC19* is likely to be involved in

antibiotic sequestration to the vacuole, it can be overexpressed for use as a marker. Once antibiotic import proteins are uncovered and characterized, additional markers may be developed via RNAi-mediated downregulation of these proteins. In fact, a recent paper describes RNAi-mediated knockdown of the gene described in this dissertation for use as a new molecular marker (Aufsatz et al., 2009).

Summary

Plants have co-existed with antibiotics for as long as they have had mutualistic relationships with the soil-dwelling, antibiotic-producing actinomycete bacteria. Additionally, plants are exposed to antibiotics whenever contaminated manure is used as fertilizer. Recent studies have shown that plants are capable of accumulating measurable levels of antibiotic, and that uptake of antibiotic seems to be an energy-dependent process. However, the molecular mechanisms by which plants are capable of antibiotic uptake remain unknown. Knowledge about plant transport proteins involved in uptake of antibiotic not only aids us in the development of a safer, healthier food supply, but it also provides us with new candidates for safer, plant-based molecular markers for the construction of transgenics.

Outline of Dissertation

Despite concerns over antibiotic accumulation in crop plants, very little is known about how plants are capable of uptake and distribution of antibiotic, both on a cellular level and within the plant body as a whole. This dissertation describes the discovery and

characterization of a chloroplast-localized transport protein of *Arabidopsis thaliana* with a very interesting function – the import of aminoglycoside antibiotics. To our knowledge, this protein (named MAR1, for Multiple Antibiotic Resistance 1) represents the first known example of an antibiotic import protein in a plant system. Knowledge about MAR1 contributes to our understanding of the fate of antibiotics in plants, which is currently in its infancy.

After an EMS mutagenesis experiment, a mutant was isolated that was resistant to multiple aminoglycoside antibiotics. The mutant was named *m*ultiple *a*ntibiotic *r*esistance *1* (*mar1-1*). A mapping population was generated, and the mutation was localized to the middle of a putative transmembrane domain of a previously uncharacterized transport protein. The transporter, At5g26820, was annotated as having low similarity to Ferroportin1 from *Danio rerio*, and had only two other homologs in *Arabidopsis*. Phylogenetic analysis revealed that At5g26820 was more similar to its homologs in other plant species (rice and grape), which were either chloroplast- or secretory-pathway-localized. Overexpression of At5g26820 reversed the resistance phenotype of *mar1-1*, while native expression sensitized the mutant to approximately wild-type levels. Two independent T-DNA insertions were obtained in At5g26820 (subsequently referred to as *mar1-2* and *mar1-3*). Both lines were able to phenocopy the multiple resistance phenotype of *mar1-1*. Thus, At5g26820 was named *MAR1*.

YFP fusion proteins were constructed to elucidate the subcellular localization pattern of MAR1 in *Arabidopsis* whole plants and protoplasts. The MAR1 signal peptide alone delivered YFP signal to the chloroplasts, while full length MAR1-YFP localized to

the chloroplast periphery. These data provide evidence of MAR1's role as a chloroplast-membrane-localized transport protein. When MAR1 is disrupted, antibiotics are unable to reach their ribosomal targets in the chloroplast, therefore resistance is achieved. MAR1 overexpression floods the chloroplast with antibiotic, causing hypersensitivity.

To further test MAR1's role as an aminoglycoside importer, we expressed this protein in yeast. Expression of wild-type MAR1 caused hypersensitivity to the aminoglycoside G418 at three different concentrations. Expression of mutant *mar1-1* also caused hypersensitivity, albeit to a lesser extent than the wild-type transporter. This data provides evidence that MAR1 is importing antibiotic, and also seems to indicate that the *mar1-1* mutation causes a decrease in function of the transport protein.

To test MAR1's role as a chloroplast import protein, we performed aminoglycoside uptake assays using isolated chloroplasts and whole seedlings. Using these assays, we were able to show that *mar1-1* mutant chloroplasts accumulate less gentamicin than wild-type, while MAR1 overexpressors accumulate more than wild-type. Mutants (*mar1-1* and *mar1-3*) also accumulated less gentamicin than wild-type in whole seedling assays. Taken together with previous experiments, these uptake experiments have allowed us to develop a model for MAR1 function as an antibiotic import protein on the chloroplast envelope.

It is unlikely that evolutionary pressures would have selected for a means of entry for toxic antibiotics into plant chloroplasts. Therefore, we propose that *MAR1* has a more "conventional" role in the plant, and the transport of antibiotics is an opportunistic effect. Given its sequence similarity to ferroportin, it is possible that *MAR1* could be involved in

some aspect of iron transport. Interestingly, we observed a visible and quantifiable phenotype of chlorosis, a common symptom of iron deficiency, in 35S::*MAR1* seedlings when grown on MS plates for two weeks. Additionally, when 35S::*MAR1* plants were grown in soil, we observed a prominent chlorosis phenotype along the midvein and older areas of cauline leaves. Chlorosis of plate-grown seedlings persisted until media was supplemented with 300mM Fe-EDTA, suggesting that the overexpression of *MAR1* creates an iron-limiting condition for the plant.

We also examined *MAR1* for transcriptional changes under iron limitation and iron excess. A 60% decrease in *MAR1* expression was observed after 4 days of growth under iron deficiency (300mM ferrozine), and this downregulation was also observed when plants were grown for two weeks on plates containing a lower concentration of ferrozine (100mM). A subsequent increase in *MAR1* transcription was not observed when iron levels were elevated for four days. Since the chlorosis of 35S::*MAR1* can be rescued by excess iron, and *MAR1* is downregulated under limiting iron conditions, we postulate that *MAR1* may play a role in iron chelation, storage, or sequestration.

CHAPTER II

Results and Discussion: Mapping and Cloning of an *Arabidopsis thaliana* Mutant Resistant to Multiple Aminoglycoside Antibiotics

Isolation of an Antibiotic Resistant Mutant via EMS Mutagenesis

An antibiotic resistant mutant of *Arabidopsis thaliana* (*m*ultiple *a*ntibiotic *r*esistance 1; *mar1-1*) was isolated via ethyl methane sulfonate (EMS) mutagenesis on an *Arabidopsis* transgenic line (ecotype Landsberg *erecta* or L*er)* harboring a methylation-silenced Nopaline Synthase (Nos) promoter-NPTII transgene. Nos-NPTII is normally constitutively expressed and confers kanamycin resistance to plant cells, but this silenced line was kanamycin sensitive. This mutagenesis was originally designed to uncover genes in the methylation pathway responsible for the NPTII silencing by screening for kanamycin resistant mutants. Several kanamycin resistant mutants were successfully recovered from the screen. For one of these mutants, outcrossing to wild type and subsequent segregation revealed that the resistance was a single-locus nuclear trait. Furthermore, the resistance did not cosegregate with the transgene on the Transferred DNA (T-DNA). Thus, the mutation had nothing to do with the original NPTII locus. One of the kanamycin resistant, T DNA-minus segregants from this line was crossed to the Columbia ecotype (Col) to generate a mapping population.

mar1-1 Displays Multiple Antibiotic Resistance

The mutant described above, *mar1-1*, is resistant to multiple antibiotics at varying concentrations. In addition to kanamycin, it is also resistant to tobramycin, gentamicin, streptomycin, amikacin and apramycin (Figure 2.1 A). All of these antibiotics belong to the aminoglycoside class, and therefore have great structural and functional similarity. Structurally, all aminoglycosides consist of several aminated sugars joined in glycosidic linkages to a dibasic cyclitol (Figure 2.2 A and B). Functionally, they inhibit prokaryotic protein synthesis by binding the 30S ribosomal subunit, which perturbs elongation of nascent polypeptide chains and also impairs the proofreading process. This leads to misreading and/or premature termination of translation (Mingeot-Leclercq et al., 1999). The resistance of *mar1-1* is highly specific for aminoglycosides that target prokaryotic translational machinery and does not extend even to the structurally similar aminocyclitol, spectinomycin (Figure 2.1 D; for the structure of spectinomycin see Figure 2.2 C)

There are a few aminoglycosides (specifically, G418, paromomycin and hygromycin; Figure 2.2 B) which are additionally capable of inhibiting eukaryotic protein synthesis (Eustice and Wilhelm, 1984). G418 is able to bind directly to the 80S ribosomal complex (Mingeot-Leclercq et al., 1999) and inhibits elongation, paromomycin induces misreading and readthrough of stop codons (Fan-Minogue and Bedwell, 2008), and hygromycin also induces misreading (Eustice and Wilhelm, 1984). Since G418 and hygromycin do not cause chlorosis in plants, we were not able to do chlorophyll assays to determine resistance/sensitivity of *mar1-1*. However, we tested a wide range of

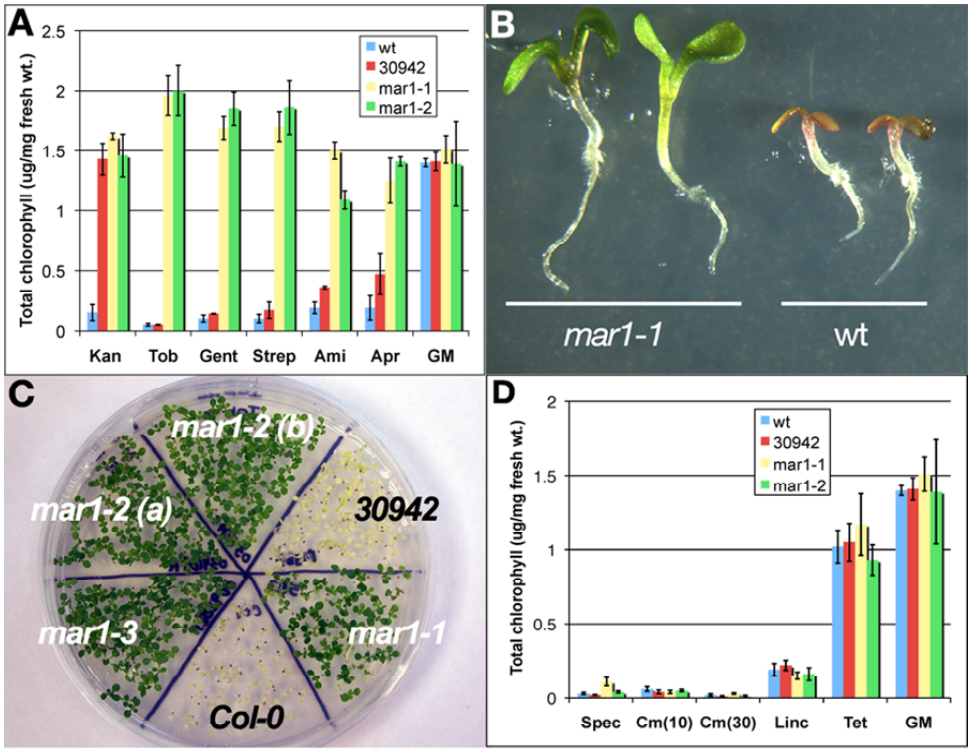

Figure 2.1. Resistance Phenotypes of *mar1-1*, *mar1-2*, and *mar1-3*. **(A)** Chlorophyll content of seedlings grown on aminoglycoside antibiotics. Wild type (wt) seedlings and an unrelated homozygous T-DNA line, Salk_030942 (30942), were used as controls. Antibiotic concentrations were: kanamycin (Kan) 25 mg/L, tobramycin (Tob) 40 mg/L, gentamicin (Gent) 70 mg/L, streptomycin (Strep) 75 mg/L, amikacin (Ami) 100 mg/L, apramycin (Apr) 200 mg/L. GM was plain growth media (no antibiotic). **(B)** Phenotypes of seedlings grown on MS media + kanamycin (25 mg/L) for 7 days. **(C)** Phenotypes of the Salk T-DNA knockout mutants *mar1-2* (two individual homzygotes are indicated as (a) and (b)) and *mar1-3*, along with control line (30942) and Col-0, grown on MS media + tobramycin (40 mg/L) for 14 days. **(D)** Chlorophyll content of seedlings grown as in **(A)** on media containing four non-aminoglycoside antibiotics. Antibiotic concentrations were: spectinomycin (Spec) 8 mg/L, chloramphenicol (Cm) 10 mg/L and 30 mg/L, lincomycin (Linc) 25 mg/L, tetracycline (Tet) 10 mg/L. GM was plain growth media (no antibiotic).

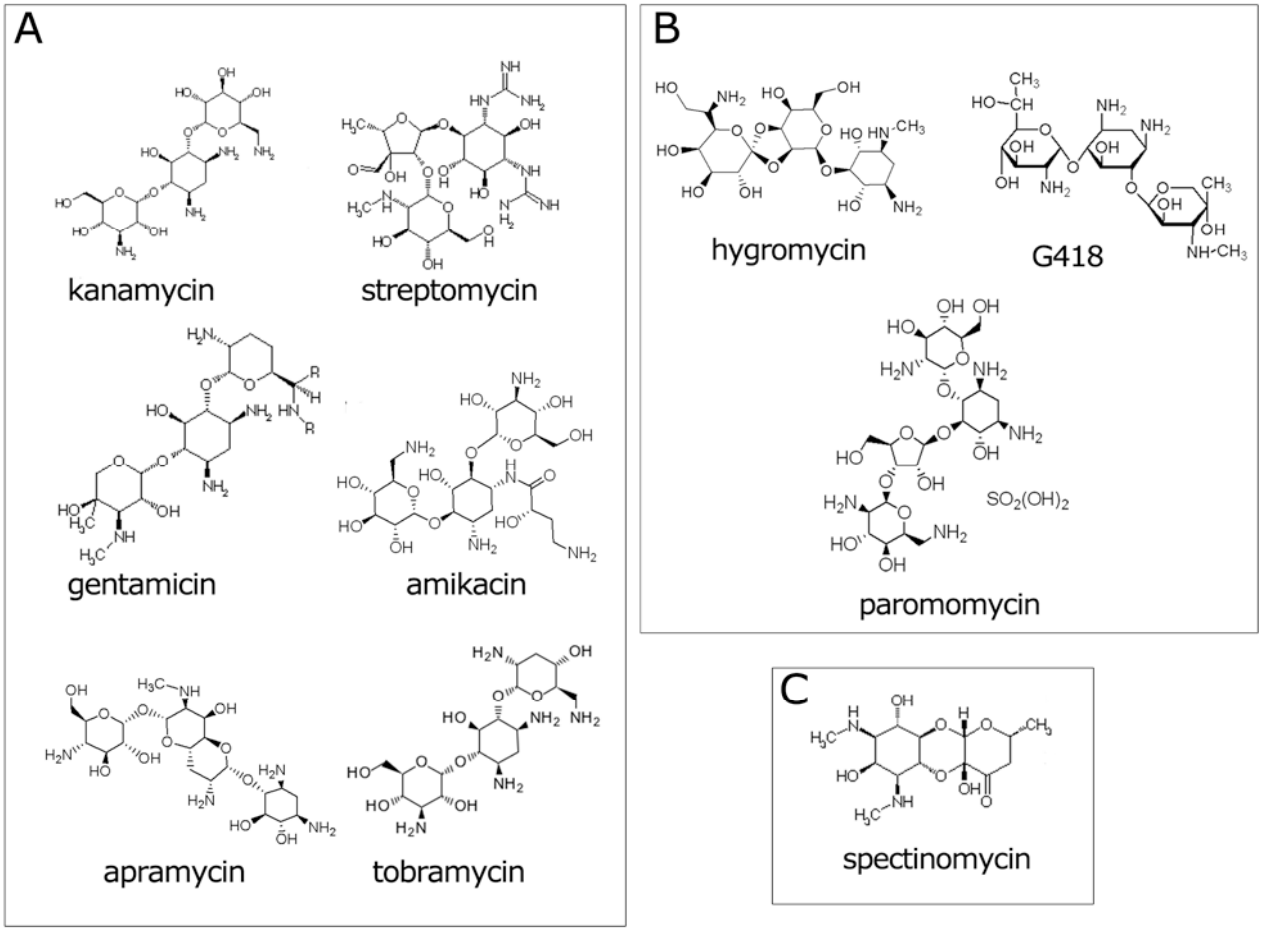

Figure 2.2. Representative Structures of Aminoglycosides and the Structurally Related Aminocyclitol, Spectinomycin. The basic aminoglycoside structure consists of a dibasic cyclitol conjugated to multiple aminated sugars. **(A)** Aminoglycosides known to inhibit prokaryotic translation. **(B)** Aminoglycosides known to inhibit both prokaryotic and eukaryotic translation. **(C)** The structurally related aminocyclitol, spectinomycin, which lacks the aminated sugar moiety common to all aminoglycosides.

concentrations and examined seedlings closely for phenotypic differences. For G418, we tested 25, 50, 100, 300, and 500 mg/L; for hygromycin, we tested 1, 2, 5, 6, 7, 8, 9, 10, 20, 30, and 40 mg/L. We saw no difference in growth between *mar1-1* and wild-type L*er* at any of these concentrations (representative images are shown in Figure 2.3). Additionally, *mar1-1* was not resistant to the aminoglycoside paromomycin and appeared just as sensitized as the wild type Col-0 (Figure 2.4), while plants expressing NPTII do show significant resistance (Figure 2.4, bottom row) due to the detoxifying effect of neomycin phosphotransferase on paromomycin (Torbert et al., 1995).

Backcrosses of individual F2 mutants isolated from the mapping population revealed that the mutation behaves as an incomplete dominant, with heterozygotes displaying an intermediate phenotype of partial resistance (Figure 2.5). This could indicate that the MAR1 protein acts as a dimer, and complexes containing a mutated subunit are less functional. Alternatively, the *MAR1* gene could be haploinsufficient, and a single functional copy of the gene is not enough to bring about the wild-type condition. The resistance can be transmitted by either male or female parent, so the mutation is nuclear.

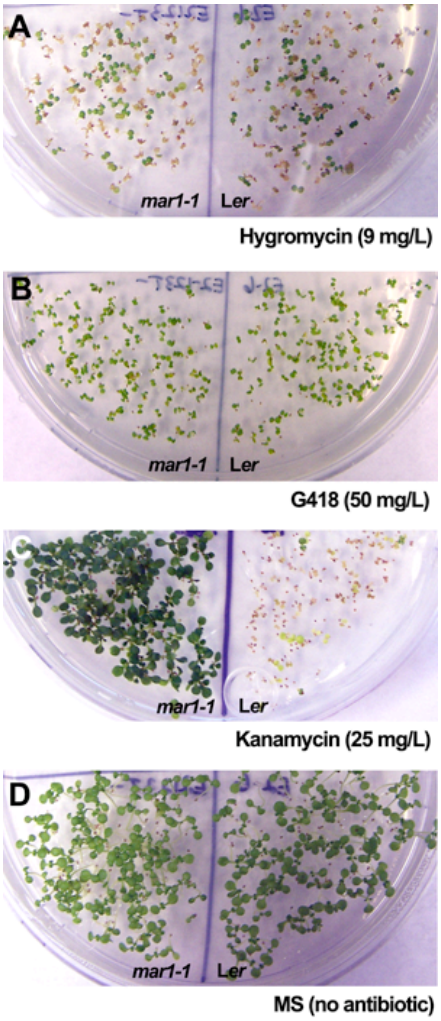

Figure 2.3. *mar1-1* is Not Resistant to the Aminoglycosides Hygromycin and G418. Seeds were surface sterilized and plated on MS plates (plus or minus antibiotic) as described in Methods. Seedlings were photographed after two weeks of growth on the indicated concentration of antibiotic. Kanamycin **(C)** and plain MS **(D)** are included as controls

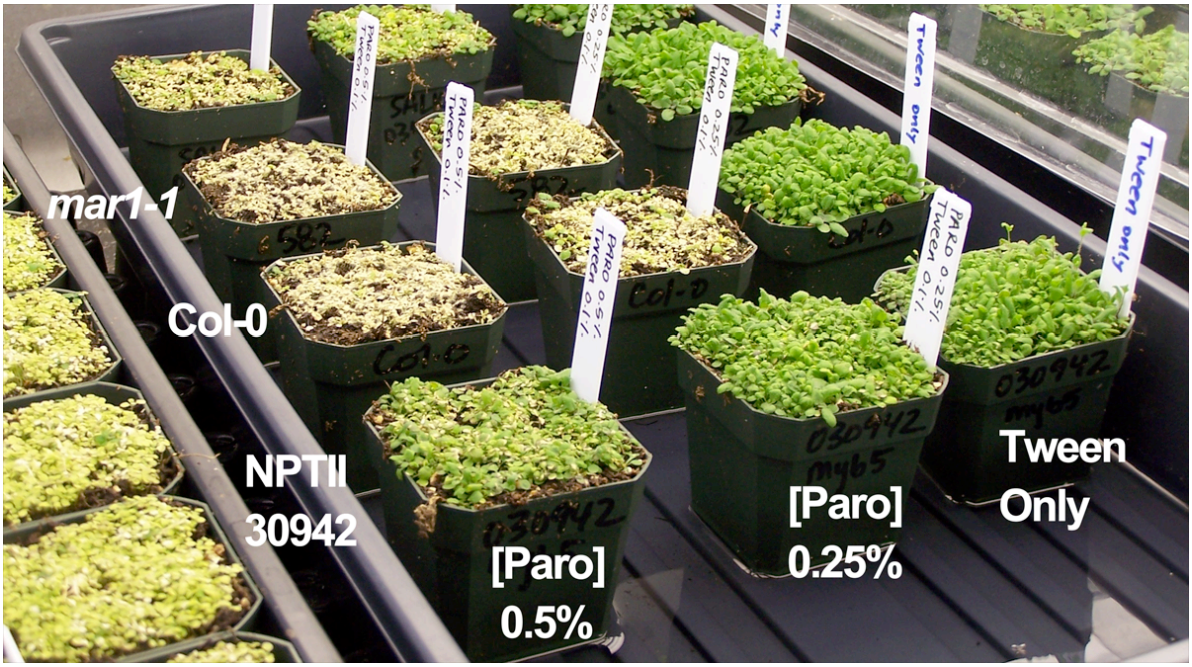

Figure 2.4. *mar1-1* is Not Resistant to the Aminoglycoside Paromomycin. Plants were grown for 20 days in soil under constant light conditions in a growth room. Approximately 25 mL of a paromomycin solution (first and second columns, concentrations as indicated) or tween-only control (third column) was sprayed on plants as indicated. Front row – Salk_030942, second row – Col-0, third row – *mar1-1*. Photos were taken 7 days after spraying. Paromomycin concentration is given as % w/v. Tween 20 is used in all solutions as a surfactant at 0.1% v/v.

Linkage Mapping of *mar1-1*

The *mar1-1* (L*er* background) X Col F2 mapping population mentioned earlier was screened for kanamycin resistance at a concentration of 25 mg/mL. 608 kanamycin resistant individuals were isolated and their DNA extracted for use in linkage mapping. Microsatellite loci polymorphic between Col and L*er* were utilized as molecular markers to crudely map the mutation to the upper arm of chromosome V. As we began to narrow down the region, we developed new polymorphic microsatellite markers (using Microsat Radar software), as well as L*er*/Col SNPs (by sequencing short regions in the area of the locus). The region containing the lesion was narrowed to 150 kB on chromosome V. This region contains 33 genes, one of which is a putative transporter (Figure 2.6). The putative transporter, At5g26820 (referred to in this manuscript text and figures as MAR1), is annotated as having low similarity to Ferroportin1, an iron exporter from *Danio rerio*. The protein sequence has no conserved domains, but has been described as *AtIREG3* based on sequence similarity to *AtIREG1* and *AtIREG2*, two iron-regulated transporters in *Arabidopsis* (Schaaf et al., 2006). More recently, it has been described as *RTS3*, and two mutations in the gene (*rts3-1* and *rts3-2*; Figure 2.7 A and B) were shown to confer kanamycin resistance at 40 mg/L (Aufsatz et al., 2009).

There are three *MAR1* homologs in rice (*Oryza sativa*; Os12g3570, Os05g04120, Os06g36450) and two homologs in grape (*Vitis vinifera*; A5AS54, A5BT51). *MAR1* is more closely related to rice homologs that are predicted to be chloroplast localized (Os12g37530 and Os05g04120), and to its grape homolog that is predicted to function in secretory pathways (A5AS54) (Figure 2.7 D). This could indicate that the MAR1 protein

may localize to an intracellular compartment, as opposed to the plasma membrane of the cell. Additionally, ARAMEMNON, the plant membrane protein database (http://crombec.botanik.uni-koeln.de/index.ep), predicts that the MAR1 protein contains 11 transmembrane spanning domains, and has a putative chloroplast transit peptide (Consensus score of 11.7; Figure 2.7 B).

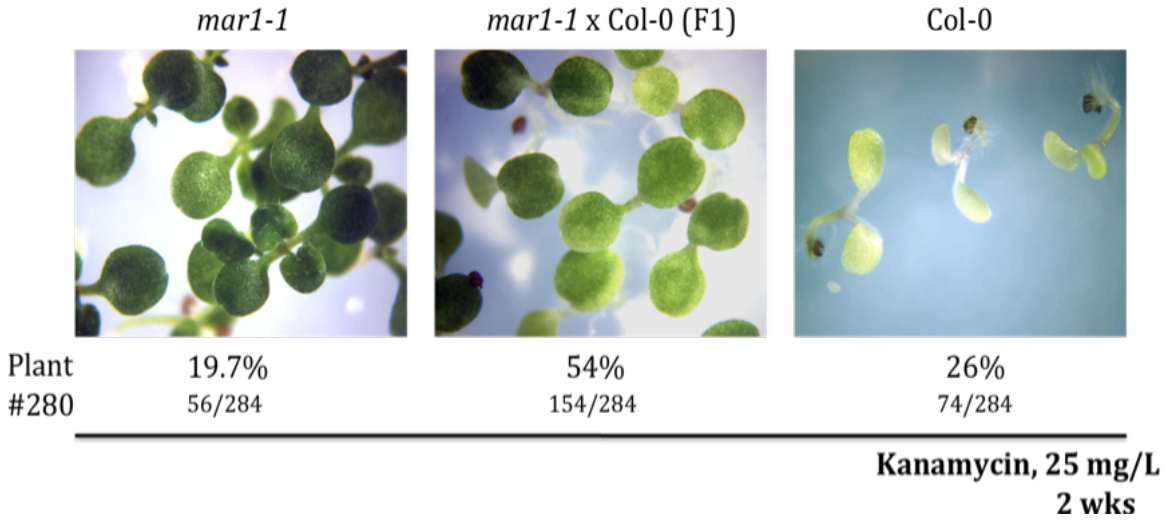

Figure 2.5. The *mar1-1* Mutation Behaves as an Incomplete Dominant. When a homozygous *mar1-1* plant was crossed to wild-type Col, the F1 displayed a phenotype of intermediate resistance when grown on kanamycin 25 mg/L (middle panel). *mar1-1* (left panel) and Col-0 (right panel) are included for phenotypic comparison. A plant genotyped as heterozygous at the *mar1-1* locus (Plant #280) produced progeny that were 19.7% phenotypically resistant (56/284 seedlings), 54% intermediate (154/284 seedlings), and 26% sensitive (74/284 seedlings).

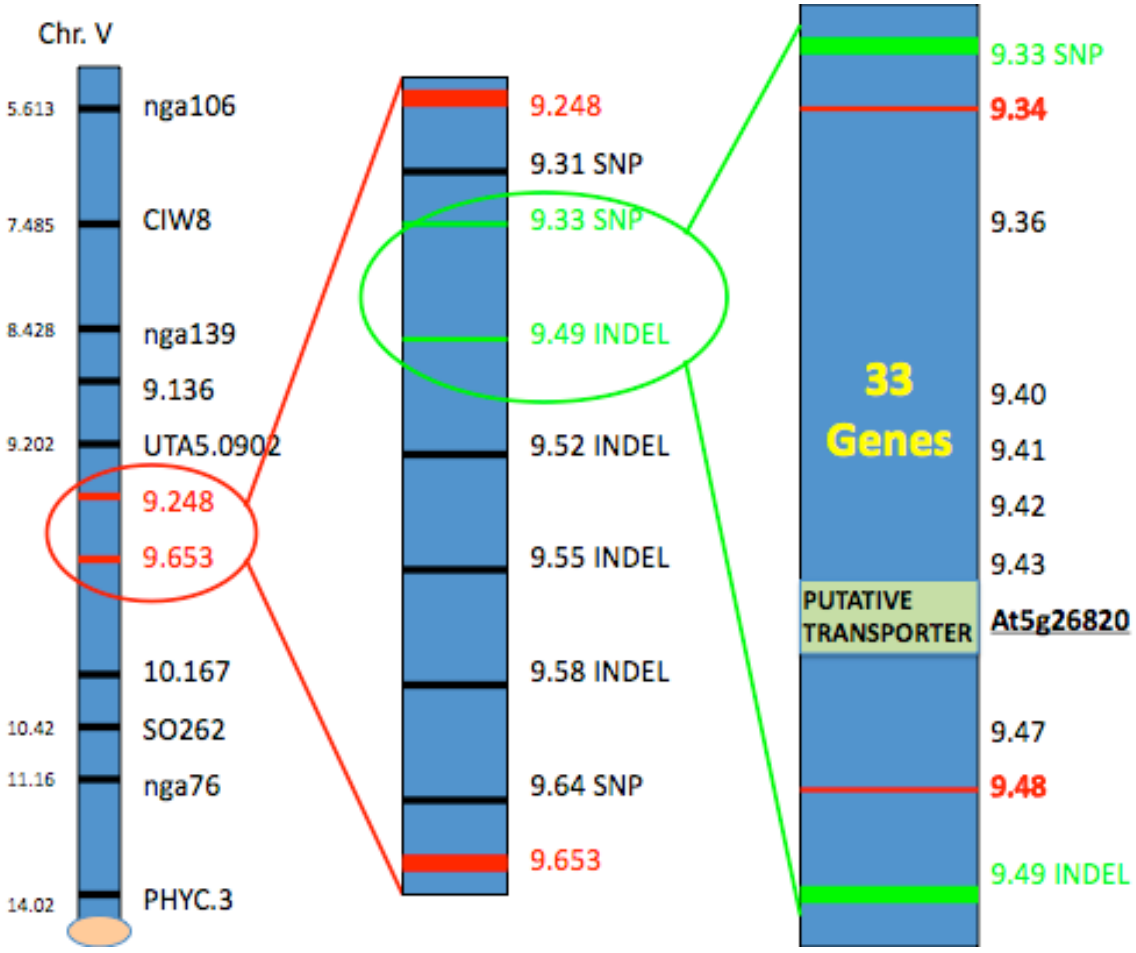

Figure 2.6. Linkage Mapping of the *mar1-1* Mutation. The mar1-1 mutation was rough-mapped using microsatellite markers (left column). Fine mapping was done using SNPs and INDELs, as indicated (middle column). The mutation was narrowed down to a 150 kB region containing 33 genes. A putative transporter (At5g26820) as isolated as a potential candidate gene (right column).

Direct sequencing of *MAR1* revealed a single nucleotide change (C to T) present in the *mar1-1* mutant parent as well as 5 individual kanamycin resistant F2 plants isolated from the mapping population. This change was not found in the L*er* parent (pre-EMS mutagenesis) or in our lab strains of wild type Col and L*er*. Additionally, it was not found in another multiply antibiotic resistant plant that was isolated from the mutagenesis screen (E2-112), also in the L*er* genetic background.

The C to T nucleotide change occurs in a predicted exon and results in a single amino acid change from alanine to valine in amino acid 441 (A441V). This change is in the middle of predicted transmembrane helix 7. Alanine 441 is conserved among *MAR1* homologs in *A. thaliana*, *Oryza sativa* (rice), and *Vitis vinifera* (grape) (Figure 2.7 C). This high level of conservation could indicate that this particular residue is very important for the function of the MAR1 protein. Homologs that do not have alanine at position 441 replace this residue with either serine or glycine (Figure 2.7 C) – two amino acids with small R-groups. It is therefore likely that the addition of two relatively bulky methyl groups at this position in the mutant protein is enough to substantially alter function.

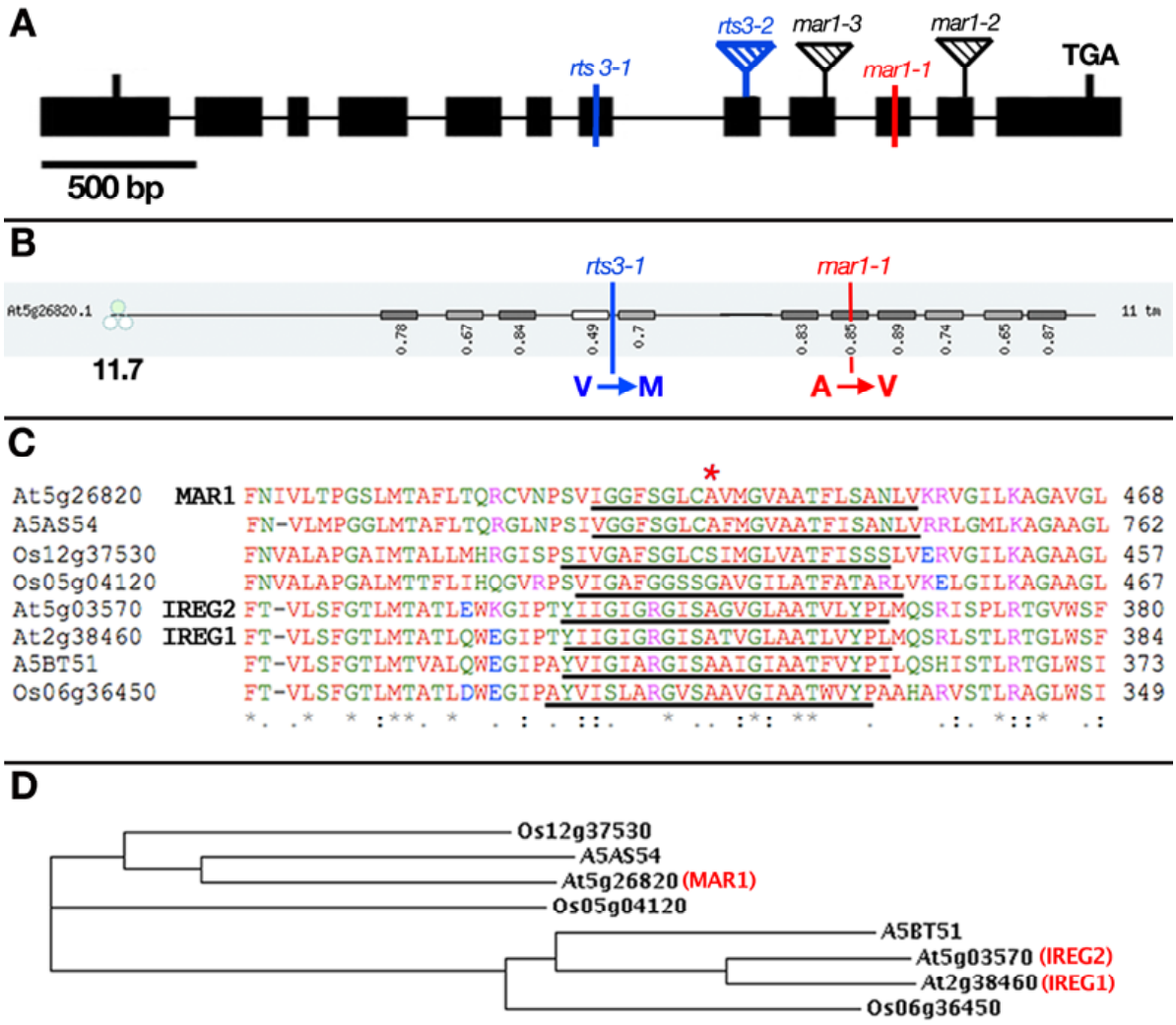

Figure 2.7. Analysis of the *MAR1* Gene and Protein. (A) The *MAR1* gene in *Arabidopsis*. Exons are depicted as solid black boxes. The mutation site for *mar1-1* and the insertion sites for SALK lines *mar1-2*, *mar1-3*, and GABI-KAT line *rts3-2* (Aufsatz et al., 2009) are shown. (B) Transmembrane domains in MAR1 are shown along with consensus score values (Schwacke et al., 2003). Domains with consensus scores above 0.42 are counted in the total number of transmembrane domains. A putative chloroplast transit peptide is predicted (with 11.7 consensus score value) (Schwacke et al., 2003; Schwacke et al., 2007). The amino acid changes in mutant *mar1-1* and *rts3-1* are also

shown. (C) Alignment of MAR1 (At5g26820) with its homologs in *Arabidopsis* (IREG2 (At5g03570), IREG1 (At2g38460)), *Oryza sativa* (Os12g37530, Os05g04120, Os06g36450), and *Vitis vinifera* (A5AS54, A5BT51). Degree of conservation of various amino acids is indicated below the alignment by periods (highly conserved), colons (very highly conserved), and grey asterisks (most highly conserved). Underlined areas illustrate the predicted transmembrane domains around the mutation site (as calculated by TMHMM, http://www.cbs.dtu.dk/services/TMHMM-2.0/). A red asterisk above the alignment indicates the site of the *mar1-1* mutation. (D) Phylogram of MAR1 and related proteins listed in (C). The alignment in (C) and phylogenetic tree in (D) were created using ClustalW (Larkin et al., 2007) .

The T-DNA Insertion Mutants *mar1-2* and *mar1-3* Phenocopy *mar1-1*

We obtained two T-DNA insertion lines for *MAR1* (Salk_034189 and Salk_009286) from the *Arabidopsis* Biological Resource Center. We have designated Salk_034189 as *mar1-2* and Salk_009286 as *mar1-3*. Both lines show an extreme reduction in *MAR1* transcript, as measured by quantitative real-time PCR (Figure 2.8 A), and both were found to be nearly phenotypically identical to *mar1-1*, with respect to antibiotic resistance (Figure 2.1 A and C). Note that Salk lines are expected to be kanamycin and paromomycin resistant due to expression of *nptII*, but this does not lead to cross-resistance to other antibiotics, as illustrated by an unrelated kanamycin resistant, *nptII*-expressing Salk insertion line (Salk_030942; Figure 2.1 A and C). The nearly identical phenotypes of *mar1-1*, *mar1-2*, and *mar1-3* (Figure 2.1 A and C) indicate that all alleles are probably hypomorphic mutations, and since all confer multiple resistance, the MAR1 transporter must be a means of entry for antibiotics.

Overexpression of *MAR1* Confers Multiple Antibiotic Hypersensitivity

Since the T-DNA insertion lines *mar1-2* and *mar1-3* phenocopy the EMS mutant *mar1-1*, and all mutations confer multiple antibiotic resistance, we hypothesized that overexpressing *MAR1* would lead to the opposite phenotype – hypersensitivity to multiple antibiotics. We expressed the *MAR1* genomic locus from start to stop codon under control of the strong, constitutive CaMV35S promoter in wild type plants, and found that it did, in fact, confer a phenotype of hypersensitivity to both kanamycin and gentamicin, based on severe chlorosis and stunted growth of seedlings (Figure 2.9 A and B). *MAR1* expression in two independent overexpression lines was found to be at least 48-fold higher than wild type (Figure 2.8 B)

To further confirm that mutations in At5g26820 are responsible for the phenotype of *mar1*, we also expressed 35S::At5g26820(*MAR1*) in the *mar1-1* background. Analysis of several independent transgenic lines revealed that this construct led to a reversal of the kanamycin resistance phenotype of *mar1-1*, i.e., mutant *mar1-1* plants overexpressing *MAR1* were found to be hypersensitive to kanamycin (Figure 2.10). Additionally, native expression of *MAR1* in a *mar1-2* background reverted the phenotype back to approximately wild-type levels of resistance (Figure 2.11).

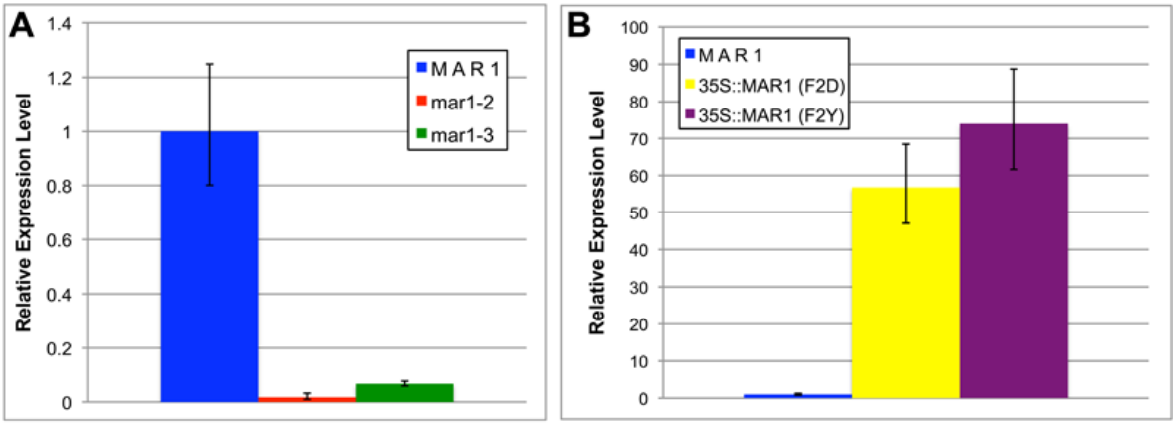

Figure 2.8. Levels of *MAR1* Expression in T-DNA Lines (*mar1-2* and *mar1-3*) and 35S::*MAR1* Overexpression Lines (F2D and F2Y). Gene expression levels were measured by relative quantitative RT-PCR. The results were calculated using the comparative Ct method (ABI bulletin) and presented as fold changes compared with wild type (indicated here as MAR1). All values were standardized to the level of actin expression in each line. Error bars indicate the ranges of expression change.

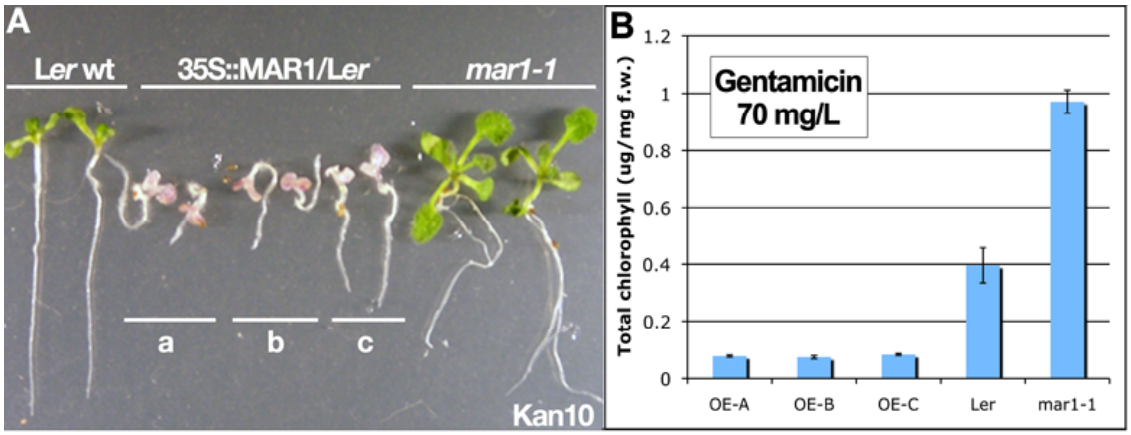

Figure 2.9. ***MAR1*** **Overexpression in L*er* Results in Hypersensitivity to Aminoglycosides.** Seeds were plated on kanamycin (10 mg/L). After 14 days, two representative seedlings of each line were photographed. Phenotypes of three independent overexpression lines are shown (a, b and c). All lines are in the L*er* background. (B) Chlorophyll content (µg of chlorophyll per mg fresh weight) of *MAR1* overexpression lines grown for two weeks on media containing gentamicin (70 mg/L). OE-A, OE-B, and OE-C are three independent *MAR1* overexpression lines. Measurements represent the average chlorophyll content (±SD) of three separate batches of seedlings

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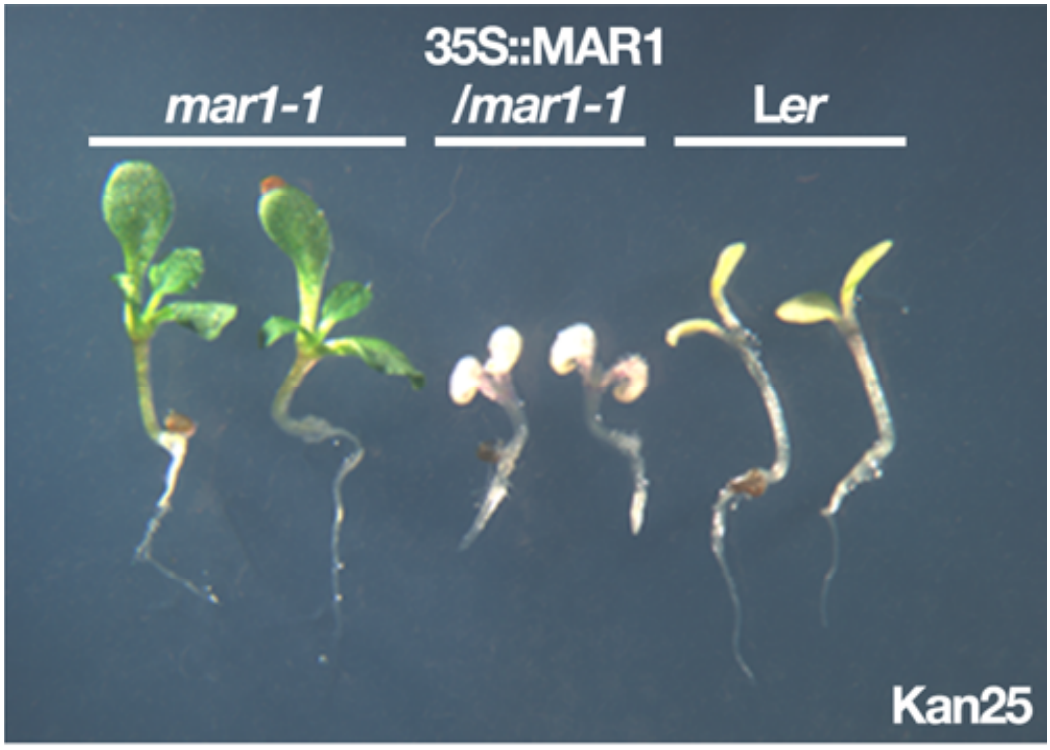

Figure 2.10. Overexpression of *MAR1* in *mar1-1* Background Reverses the Kanamycin Resistance Phenotype of *mar1-1*. Mutant *mar1-1* plants were transformed with 35S::*MAR1* and seeds were plated on kanamycin (25 mg/L). After 14 days of growth, two representative seedlings were photographed.

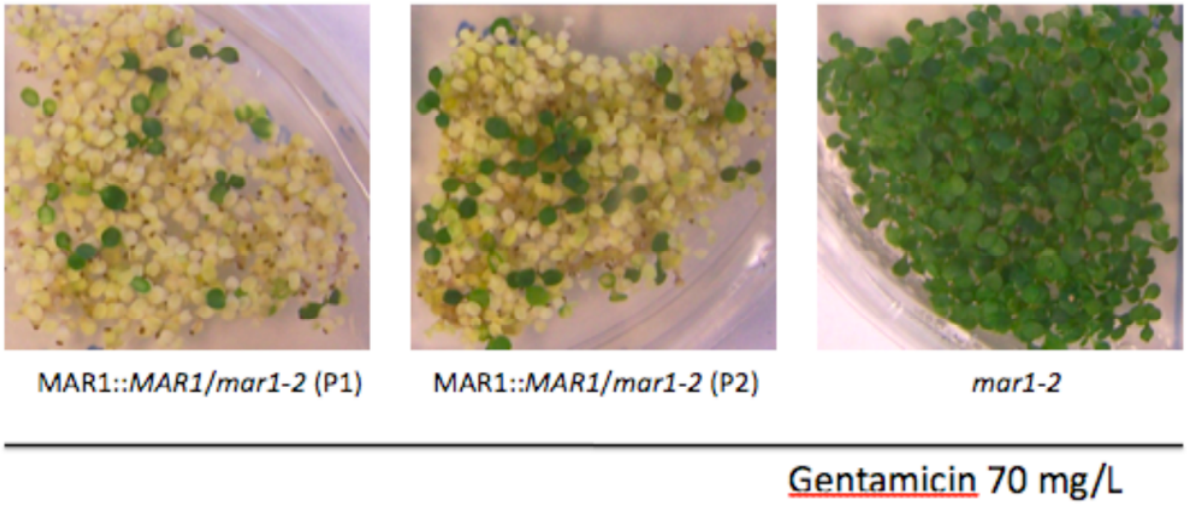

Figure 2.11. Native Expression of *MAR1* Complements the Gentamicin Resistance Phenotype of *mar1-2*. Panels 1 and 2 display seedlings from two individual Basta resistant transformants, panel 3 displays homozygous *mar1-2* seedlings. All seedlings were grown on MS media containing gentamicin (70 mg/L) for two weeks before photographing.

CHAPTER III

Results and Discussion: Characterization of the MAR1 protein – Evidence for Function as a Chloroplast-localized Antibiotic Import Protein

MAR1 Localizes to the Chloroplast Envelope

Since *mar1* mutants are sensitive to aminoglycoside antibiotics that act in the cell cytoplasm (hygromycin, G418, and paromomycin; Figure 2.3 and 2.4) but resistant to those that act only in the chloroplast (kanamycin, tobramycin, gentamicin, streptomycin, amikacin, and apramycin; Figure 2.1 A), we might predict that these mutations act to keep antibiotics out of the chloroplast. The ARAMEMNON plant membrane protein database (Schwacke et al., 2003) utilizes data from 17 individual programs to arrive at a consensus prediction for subcellular location. This consensus prediction method (Schwacke et al., 2007) predicts that the MAR1 protein is targeted to the chloroplast (consensus score of 11.7; Figure 2.7 B). The chloroplast transit peptide of MAR1 includes the first 54 amino acids of the protein, according to the ChloroP program (Emanuelsson et al., 1999). With this in mind, we created a C-terminal YFP fusion to the putative transit peptide of MAR1 for transient expression in *Arabidopsis* protoplasts. Chloroplast transit peptides are known to effectively mediate transport across the chloroplast membrane (Keegstra and Froehlich, 1999; Abdel-Ghany et al., 2005), so the expected localization of YFP fused to a transit peptide would be the stroma. This is, in

fact, what was observed in our experiment, based on distinct YFP colocalization with red (autofluorescent) chloroplasts (Figure 3.1 F, G, H).

We also constructed C-terminal and N-terminal translational fusions between full-length *MAR1* cDNA and YFP, which were used for transient expression. In C-terminal fusions, YFP fluorescence was clearly associated with chloroplasts (Figure 3.1 J, K, L), and in N-terminal fusions, fluorescence was cytoplasmic (Fig. 3.1 O, P). Since the signal peptide is the site of specific interactions with TIC (Translocon at the Inner envelope membrane of Chloroplasts) and TOC (Translocon at the Outer envelope membrane of Chloroplasts) complexes of the chloroplast envelope, it is likely that the addition of a bulky YFP fluorophore ahead of this domain may interfere with these interactions, which are necessary for import (Qbadou et al., 2003; Dixit et al., 2006). Although we have yet to experimentally confirm whether MAR1 localizes to the inner or outer membrane of the chloroplast, the presence of an N-terminal transit peptide indicates that MAR1 is likely to localize specifically to the inner envelope, since most plastid proteins of the outer envelope do not possess these transit peptides (Moller, 2005).

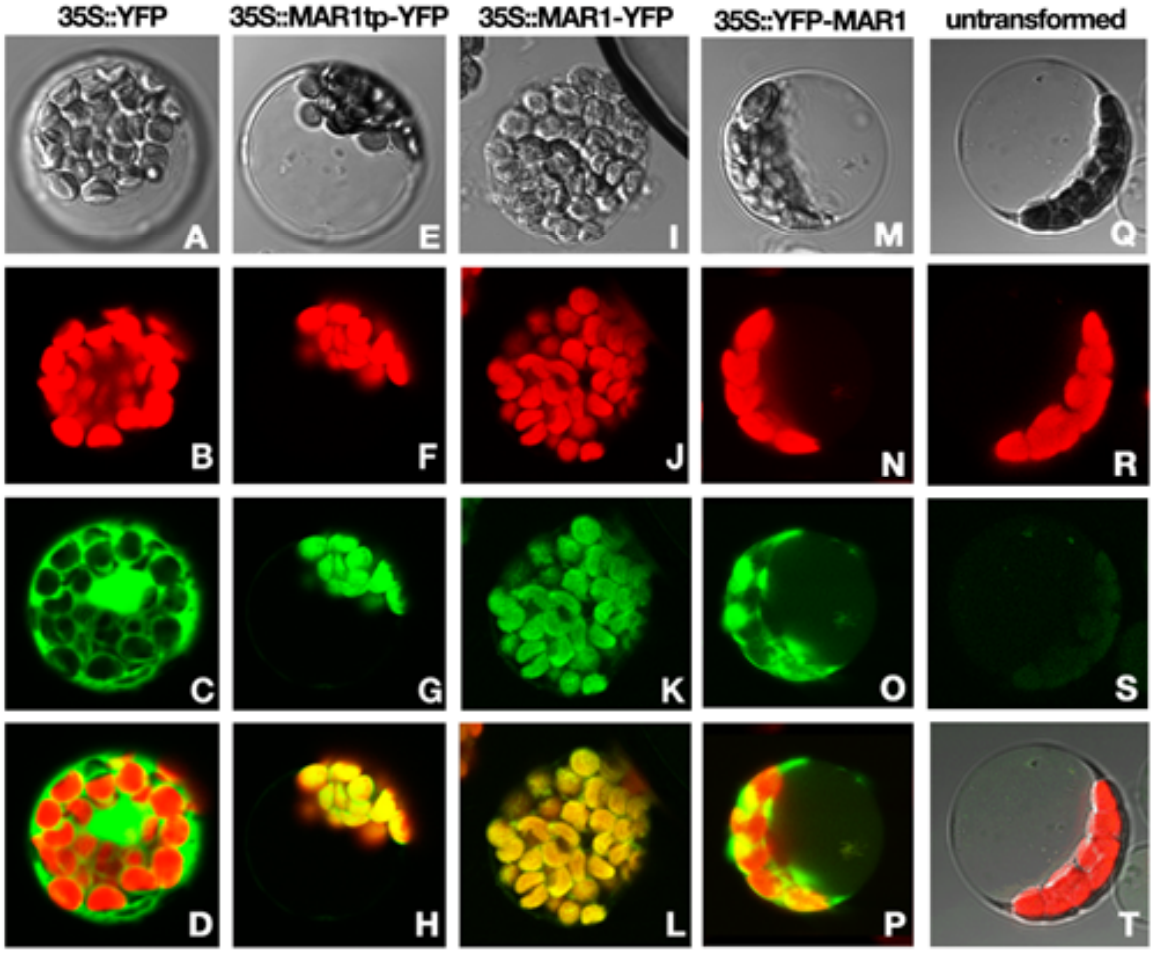

Figure 3.1. 35S::MAR1-YFP Localizes to Chloroplasts in Protoplasts. Confocal microscopic images depict the localization of YFP alone under control of the CaMV35S promoter (35S::*YFP*; first column), *MAR1* chloroplast transit peptide fused to YFP (35S::*MAR1tp-YFP*; middle column), full-length *MAR1* cDNA with YFP at the C-terminus (35S::*MAR1-YFP*; third column), full-length *MAR1* cDNA with YFP at the N-terminus (35S::*YFP-MAR1*; fourth column), and an untransformed protoplast (fifth column). A bright field image (A, E, I, M, Q), Chlorophyll autofluorescence (B, F, J, N, R), YFP fluorescence (C, G, K, O, S), and a merge of the two channels (D, H, L, P) are included for each protoplast. We note that (T) is a merge of all three channels (transmitted, chlorophyll, and YFP)

The MAR1-YFP C-terminal translational fusion described above was also used to transform plants. Both native and 35S expression of this fusion protein was able to complement the resistance phenotype of *mar1-2* (data not shown). Leaves of these plants were examined by confocal microscopy (Figure 3.2 A – L), and compared to untransformed controls (Figure 3.2 M, N, O). YFP fluorescence in transformed lines colocalized with chloroplast autofluorescence (Figure 3.2 C, F, I, L), and appeared particularly enhanced at the periphery of these organelles, indicating that MAR1 may be associated with the chloroplast envelope.

Expression of *MAR1* in Yeast Confers Hypersensitivity to the Aminoglycoside, G418

The yeast *Saccharomyces cerevisiae* is a very powerful tool for studying specific *Arabidopsis* transport proteins. Indeed, much of what we know regarding the molecular basis of plant transport has been gained via complementation strategies of yeast mutants (Barbier-Brygoo et al., 2001). The common *S. cerevisiae* lab strain, BY4700, is only slightly sensitive to an aminoglycoside that acts on both prokaryotic and eukaryotic ribosomes (G418; authors observation). Thus, to test the possible antibiotic import capability of the MAR1 putative transport protein, we expressed this protein in the yeast strain BY4700 under control of the strong PGK promoter.

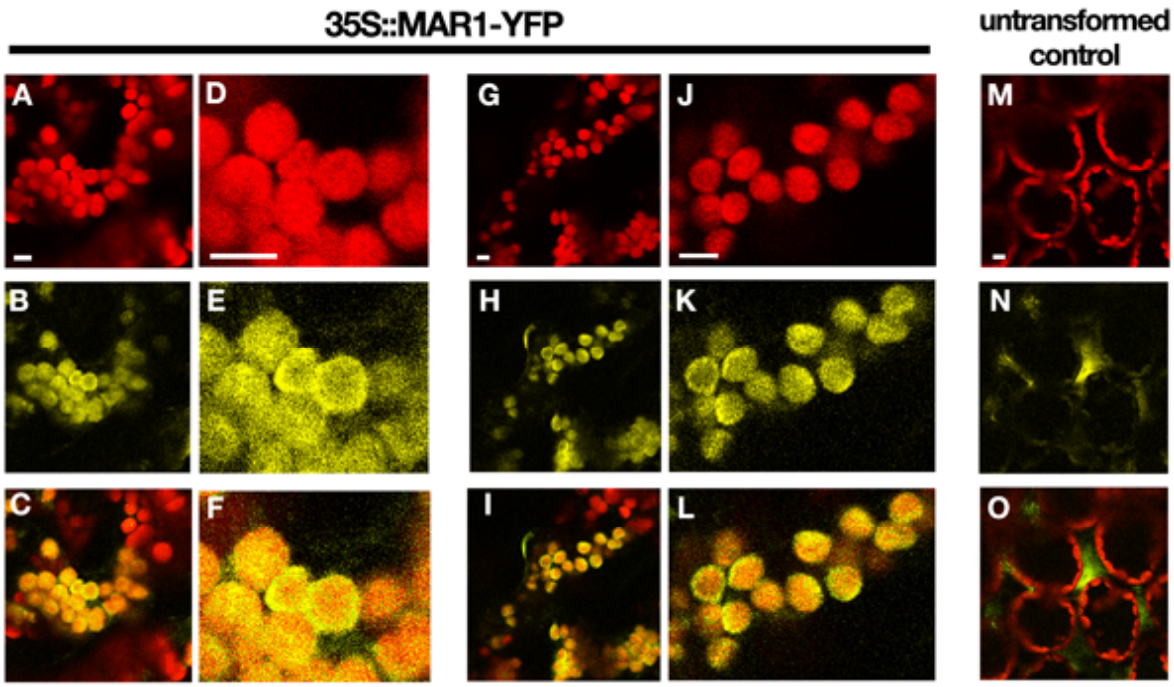

Figure 3.2. 35S::MAR1-YFP Localizes to Chloroplasts in Leaves of Transformed Plants. Plants were transformed with the C-terminal fusion construct 35S::*MAR1-YFP* as described in Methods. Confocal single-slice images of the leaves of two individually transformed plants (A – F and G – L) were compared to an untransformed leaf (M, N, O). (D, E, F) and (J, K, L) are close-up images of (A, B, C) and (G, H, I), respectively. Chlorophyll autofluorescence (A, D, G, J, M), YFP fluorescence (B, E, H, K, N), and a merge of the two channels (C, F, I, L, O) are shown for each leaf section. Scale bar = 8 μM.

Both wild-type and *mar1-1* mutant alleles were utilized for these experiments. Yeast expressing wild-type MAR1 were found to be hypersensitive to G418 when compared to empty vector controls (Figure 3.3 A). Interestingly, yeast expressing the mutant allele *mar1-1* were also hypersensitive, but to a lesser extent than the MAR1 yeast (Figure 3.3 A). To eliminate the possibility that this result was due to a general toxicity effect, the experiment was repeated using varying concentrations of cycloheximide (CHX), which is highly toxic to yeast. No growth differences were seen, at any CHX concentration, among yeast expressing either MAR1, *mar1-1*, or empty vector controls (Figure 3.3 B).

We hypothesize that the A to V mutation in mar1-1 causes a structural change in the transporter, such that its function is reduced. This reduced ability to function could be due to many factors, including reduced ability of the mutant transporter to bind or release substrate, or reduced ability to bind or release a co-transported ion (such as Na+ or H+) used as an energy source for transport. Future experiments will enable us to distinguish between these and other possibilities.

To ensure that MAR1 protein was being properly expressed, and to determine its localization pattern in yeast, we also expressed a GFP tagged version of MAR1. While GFP alone was clearly cytoplasmic (Figure 3.4 A), GFP-tagged MAR1 localized to the yeast mitochondria (Figure 3.4 B), which is typical for chloroplast membrane proteins expressed in yeast (Versaw and Harrison, 2002; Jeong et al., 2008). Since the aminoglycoside G418 acts on both prokaryotic and eukaryotic ribosomes (Vicens and Westhof, 2003), G418 is likely to be inhibiting yeast growth in MAR1-expressing strains

by accumulating in mitochondria. These experiments provide further evidence that MAR1 is capable of importing aminoglycoside antibiotic.

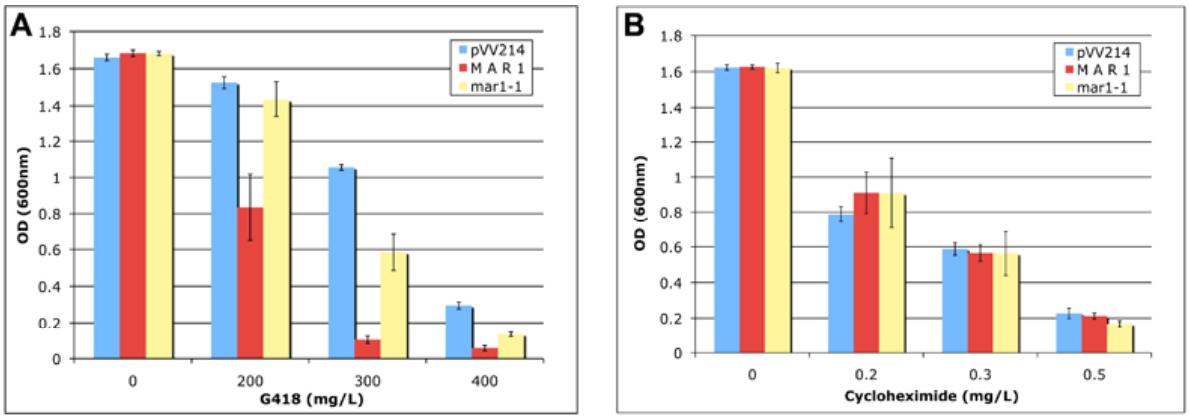

Figure 3.3. Expression of *MAR1* in Yeast Confers Hypersensitivity to G418. *MAR1* and *mar1-1* were expressed in yeast under control of the PGK promoter (vector pVV214). Cultures were standardized to OD 0.01 at 600 nm before addition of antibiotic ((A) G418 at 0, 200, 300, and 400 mg/L; (B) Cycloheximide at 0, 0.2, 0.3, and 0.5 mg/L). Cultures were analyzed spectrophotometrically after 48 hours of growth, and ODs were plotted. For both graphs, each bar represents the average absorbance of three independent cultures (±SD).

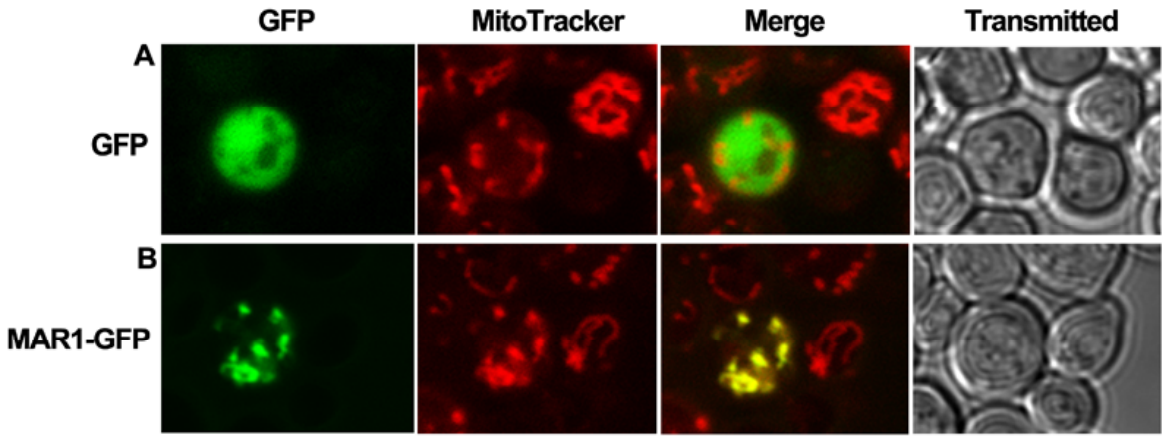

Figure 3.4. MAR1 Localizes to Mitochondria in Yeast. Yeast strain BY4700 was transformed using EGFP alone (A) or *MAR1* cDNA (lacking a stop codon) fused in-frame with EGFP (B). MitoTracker Red was used to visualize mitochondria, and a transmitted image is included to illustrate integrity of the cells.

MAR1 Regulates Gentamicin Entry into Chloroplasts

Since MAR1 appeared to be a chloroplast-localized transport protein, and its disruption and overexpression caused antibiotic resistance and hypersensitivity, respectively, we decided to test its functionality as a transporter for antibiotics. To date, there has been no research undertaken to study the uptake of aminoglycoside antibiotics in a plant system, and therefore no convenient assay was available. The assay we have developed (Figure 3.5) allows for inexpensive, non-radioactive detection of antibiotic, and is based on the ability of aminoglycosides to adsorb onto nitrocellulose membrane without the need for fixation (Mihelic-Rapp and Giebel, 1996). We developed both a short-term uptake assay using isolated chloroplasts and a longer-term uptake assay using whole seedlings. For short-term uptake, isolated chloroplasts were exposed to high levels of antibiotic (12.5 mg/mL) for short periods of time (1 and 5 min) (Figure 3.6 A). For longer-term uptake, whole seedlings were exposed to lower levels of antibiotic (70 mg/L) for two days (Figure 3.6 D). Excess antibiotic was washed away, and chloroplasts were lysed to release their antibiotic content. Lysates were then spotted onto nitrocellulose in dot-blot fashion (Figure 3.6 C) along with gentamicin standards (Figure 3.6 B), and gentamicin was detected via anti-gentamicin antibody. This allowed for a simple yet quantitative method for measuring the gentamicin content of chloroplasts – each dot was analyzed using the integrated density function of ImageJ64 to determine a relative intensity value, which correlated positively with amount of antibiotic in the lysate.

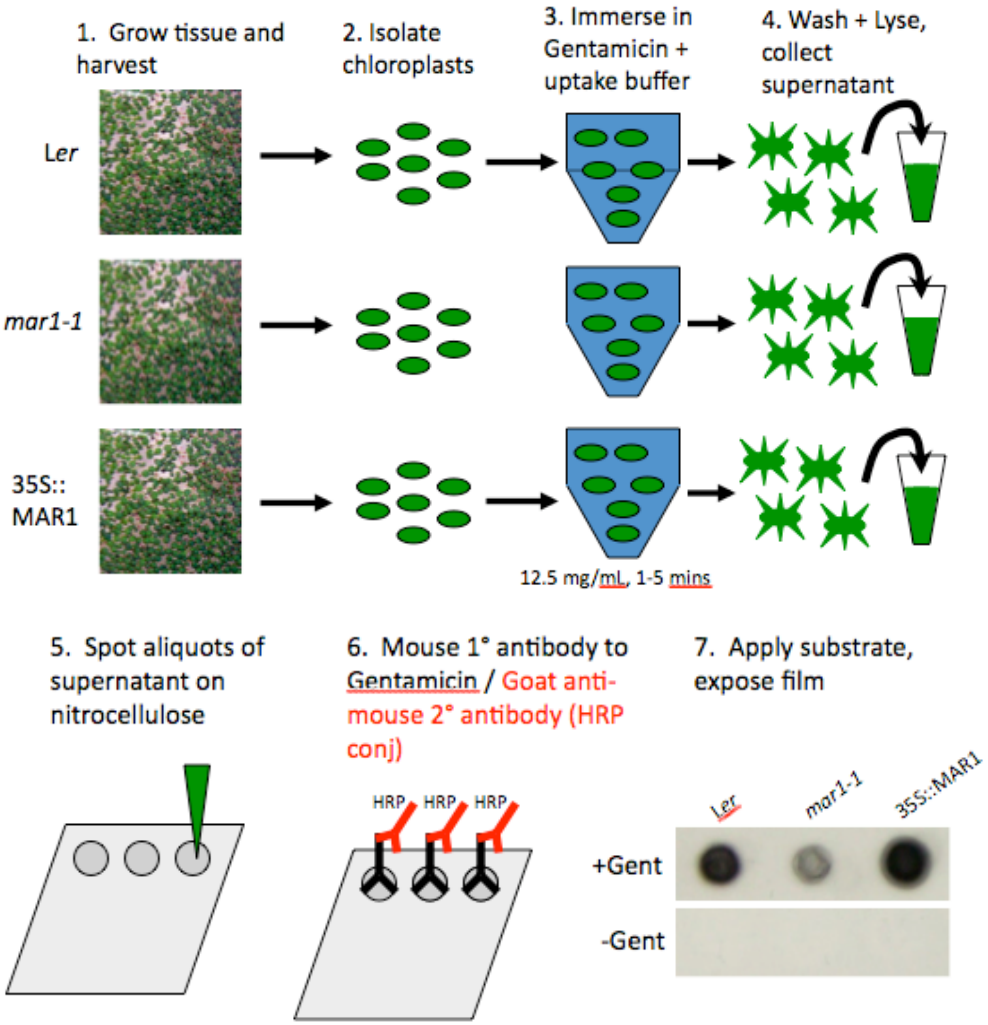

Figure 3.5. Experimental Design of the Antibiotic Uptake Experiment. For a detailed description, see methods.

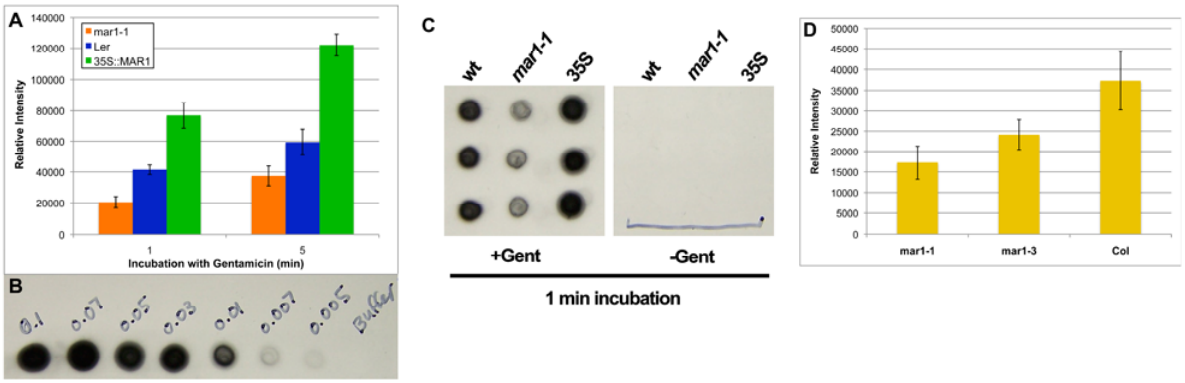

Figure 3.6. MAR1 Regulates Gentamicin Entry into Chloroplasts. (A) Plants were grown for 15 days before chloroplast isolation, and 8.5 x 10^7 chloroplasts were incubated in 12.5 mg/mL gentamicin for each uptake reaction (1 minute and 5 minutes). (B) Gentamicin standards (dissolved in chloroplast lysis buffer) were spotted as positive controls for every dot blot. Numbers above each dot indicate gentamicin concentration in mg/mL. (C) Representative data from a 1 minute uptake experiment. Left panel – triplicate lysate spots from chloroplasts incubated with 12.5 mg/mL gentamicin for 1 minute (+Gent). Right panel – triplicate lysate spots from chloroplasts incubated in uptake buffer alone for 1 minute (-Gent). In each panel, the left-hand column shows lysate from wild-type L*er* chloroplasts (wt), the middle column shows lysate from *mar1-1* (*mar1-1*), and the right-hand column shows lysate from 35S::MAR1 overexpressor chloroplasts (35S). (D) Whole seedling uptake results. Seedlings were exposed to 70 mg/L gentamicin for 2 days, washed, and chloroplasts isolated. 3 x 10^8 chloroplasts from each line were lysed. For (A) and (D), each bar represents the average relative intensity of three triplicate spots (±SD).

In short-term uptake experiments with isolated chloroplasts, it was found that chloroplasts from *mar1-1* mutant plants accumulated less gentamicin than wild type (L*er*) controls, while chloroplasts from *MAR1* overexpressors accumulated the most gentamicin (Figure 3.6 A and C). This experiment was performed a total of three independent times with the same result. In uptake experiments using whole seedlings, it was found that chloroplasts from *mar1-1* and *mar1-3* mutant seedlings accumulated less gentamicin than the wild type (Col) control (Figure 3.6 D). We note that background is extremely low (Figure 3.6 C, right panel) under the conditions that we describe (see Methods). Evidence from these experiments demonstrates the role of MAR1 as a chloroplast-associated transporter that is capable of importing aminoglycoside antibiotic.

Summary and Future Directions

mar1 represents an interesting example of plant antibiotic resistance that is based on the restriction of antibiotic entry into a subcellular compartment. Knowledge about this process – and other processes of antibiotic entry – could enable the production of crop plants that are incapable of antibiotic accumulation, aid in development of phytoremediation strategies for decontamination of water and soils polluted with antibiotics, and further the development of new plant-based molecular markers. The work described here also contributes to our understanding of how plants interact with the antibiotics they encounter, both in the laboratory (where aminoglycosides such as kanamycin are used heavily to select for transgenics) and in the natural environment.

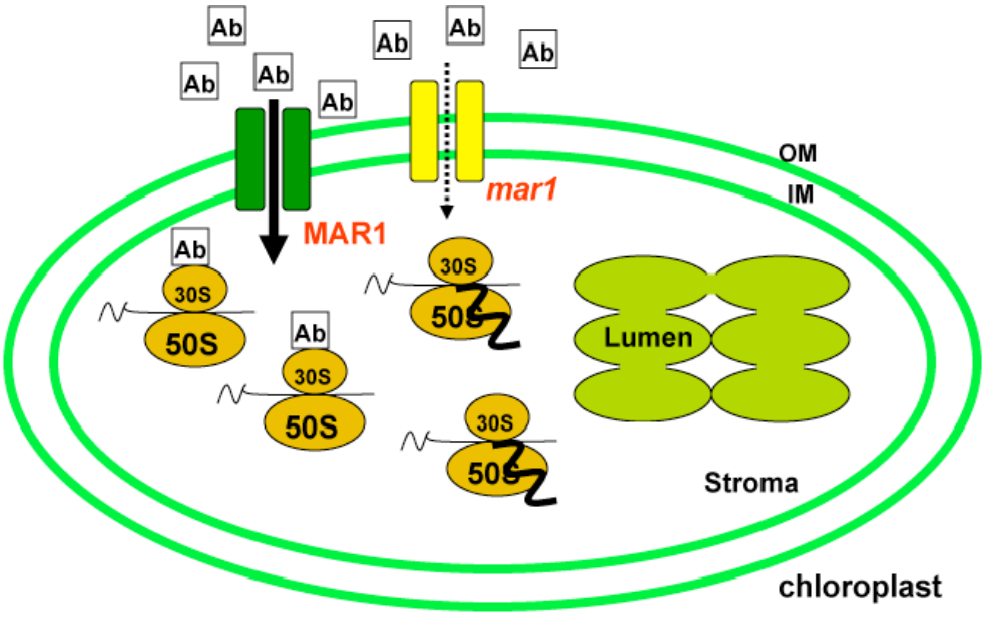

Figure 3.7. Model for Function of MAR1. Aminoglycoside antibiotics enter the chloroplast through the MAR1 transporter in order to gain access to their ribosomal targets (aminoglycosides bind the 30S ribosomal subunit where they induce misreading and/or premature termination (Recht et al., 1999)). The mutant *mar1-1* (indicated as *mar1*) is less functional, thus minimizing entry of antibiotics and conferring resistance. OM, chloroplast outer membrane; IM; inner membrane; 30S, small ribosomal subunit; 50S, large ribosomal subunit; Ab, aminoglycoside antibiotic.

A recent paper describes independent mutations of the *MAR1* locus (At5g26820) that are sufficient to achieve kanamycin resistance in *Arabidopsis* (Aufsatz et al., 2009). These findings agree with our data, however, Aufsatz *et al.* report that resistance is kanamycin-specific and does not carry over to gentamicin or hygromycin. We did not see hygromycin resistance in any of our *mar1* mutants, which was expected, because hygromycin has effects against eukaryotic ribosomes and therefore acts in the cytoplasm of the plant cell (Eustice and Wilhelm, 1984). However, we do show that *mar1* mutants are multiply resistant to several aminoglycosides, including gentamicin (Figure 2.1 A). A possible reason for this discrepancy could be that the Aufsatz *et al.* mutations are distinct from our MAR1 mutations (Figure 2.7 A and B), and thus may confer slightly different phenotypes. We also note that Aufsatz *et al.* test for gentamicin resistance at a concentration of 100 mg/L, while we test at 70 mg/L. Furthermore, Aufsatz *et al.* only mentioned the testing of kanamycin, hygromycin, and gentamicin – resistance to other aminoglycosides is not discussed.

The data presented here indicate that MAR1 is a transport protein located on the chloroplast envelope, which appears to be capable of subcellular transport of multiple aminoglycoside antibiotics (Figure 3.7). MAR1 is highly specific for aminoglycosides that act on prokaryotic translational machinery, since *mar1* mutants are not resistant to antibiotics of other classes, including those that act specifically in the chloroplast (Ellis, 1970; Kasai et al., 2004). Based on lack of sequence similarity, *MAR1* does not appear to belong to the ABC class of transporters previously implicated in *Arabidopsis* single

antibiotic resistance. Instead, MAR1 may be transporting a molecule (possibly a polyamine) involved in iron homeostasis. MAR1 is not able to distinguish between this molecule and the aminoglycosides. Further investigation is necessary to uncover the native function(s) of MAR1 in plant growth and development.

CHAPTER IV

Preliminary Studies to Uncover the Natural Function of MAR1

Introduction

The Role of Iron in Plant Metabolism

Iron is an essential micronutrient for plants because of the major roles it plays in photosynthesis, respiration, and chlorophyll biosynthesis. Iron atoms can exist in multiple redox states, and readily donate and accept electrons from their d orbitals. Thus, iron acts as an important cofactor for components of the electron transport chain, both in mitochondria and chloroplasts (Marschner, 1995). In addition, iron forms the center of Fe-S clusters, which act both as electron acceptors and donors in key plant cellular processes such as photosynthesis, respiration, sulfate assimilation and ethylene biosynthesis (Balk and Lobreaux, 2005).

Despite its essential role, levels of iron in the plant body must be tightly regulated, because it is very highly reactive. The same physical properties that allow this transition metal to act as an efficient redox cofactor and catalyst also cause it to act as a potent toxin. For example, many reactions that take place within the plant cell use molecular oxygen as an electron acceptor, thus producing superoxide ($O_2^{\bullet -}$) or hydrogen peroxide (H_2O_2) (Hell and Stephan, 2003). Although these molecules are not particularly harmful on their own, they can react with iron (via the Fenton reaction, Figure 4.1) to generate the

extremely reactive hydroxyl radical ($OH^{\bullet}$), which is highly non-selective and can react with almost every molecule found in living cells, including DNA, proteins, lipids, and sugars (Hell and Stephan, 2003). Thus, iron must be continuously bound or chelated within the cell in order to prevent oxidative damage that can arise due to Fenton chemistry.

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

$$\text{Summarized} : O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot}$$

Figure 4.1. The Fenton Reaction. Iron acts as a catalyst to convert hydrogen peroxide into a hydroxide ion and a hydroxyl free radical. The hydroxyl free radical can then oxidize organic molecules, which can result in damage to cellular components.

Iron Acquisition from the Soil

The primary source of iron for the plant is the soil. However, even when abundant, iron can be inaccessible due to its relative insolubility and tendency to form Fe hydroxides, especially at neutral or slightly basic pH. Plants attempt to overcome this by using the ATPase activity of transporters – possibly those in the AHA (*Arabidopsis* H^+ ATPase) family – to expel protons into the rhizosphere, thus decreasing the pH of the soil and releasing Fe^{3+} from insoluble oxides (Palmer and Guerinot, 2009). This can be quite effective, as one unit drop in pH increases the solubility of Fe by 1,000 fold (Guerinot and Yi, 1994).

Once freed from its insoluble hydroxide form, iron can be taken up into the cells of the plant root. The major root iron transporter in *Arabidopsis*, IRT1, has a specific affinity for ferrous (Fe^{2+}) iron (Korshunova et al., 1999; Curie and Briat, 2003). Thus, Fe^{3+} must be reduced to Fe^{2+} prior to its transport via IRT1. This reduction has been shown to be due to the action of proteins such as FRO2, which acts as a Fe^{3+}-chelate reductase in the plasmalemma of the root epidermis (Robinson et al., 1999; Hell and Stephan, 2003).

Transport of iron from root to shoot

Although the initial uptake of iron from the soil into the plant root is obviously a critical process, most of iron's essential roles occur in photosynthetic tissues. Thus, iron must be transported through the plant body from root to shoot. Ions can move easily through the

symplast from the epidermis to the pericycle, but must be actively loaded into the xylem tissues (Curie et al., 2001; Kim and Guerinot, 2007). The transporter responsible for this loading remains unknown, but considering its low solubility, iron is likely to be transported chelated to other molecules. Although both nicotianamine (NA) and citrate are capable of iron chelation, citrate appears to be the dominating Fe chelator in xylem (von Wiren et al., 1999; Green and Rogers, 2004; Durrett et al., 2007).

In the phloem, iron is thought to be transported as an NA chelate, and the transporters responsible for phloem loading and unloading are thought to be members of the YSL (Yellow Stripe Like) family of transport proteins (Haydon and Cobbett, 2007). The AtYSLs are a subgroup of the oligopeptide transporter (OPT) family in *Arabidopsis*, which were named for their ability to transport oligopeptides. Interestingly, the *Arabidopsis* protein OPT3 was demonstrated to play a role in the transport of iron (Stacey et al., 2008). Although the form in which iron was transported via OPT3 was not established, it seems likely that this Fe was chelated to NA (Palmer and Guerinot, 2009), and OPT3 has a function similar to the AtYSLs (Stacey et al., 2008).

Intracellular Iron Transport

Methods of iron acquisition from the environment have been well studied, but transporters involved in plant organellar iron transport are only recently beginning to be uncovered. In the mitochondria, iron is required for proper function of the respiratory electron transport chain, as well as for the synthesis of Fe-S clusters (Palmer and

Guerinot, 2009). However, only one transporter, STA1/AtATM3, has been implicated in the export of Fe/S clusters from mitochondria (Kushnir et al., 2001). As of yet, no mitochondrial iron importer has been identified.

The vacuole is an important metal storage compartment in seeds, and functions as an initial store of metals before uptake from the external environment is possible. The major vacuolar iron importer is VIT1, which has been shown to be essential for proper localization of iron in the seed (Kim et al., 2006). Two functionally redundant vacuolar iron exporters – NRAMP3 and NRAMP4 – have also been identified and shown to be important for iron mobilization during early seedling development (Thomine et al., 2003; Lanquar et al., 2005). The chelator nicotianamine (NA) may also play a role in sequestration of iron in vacuoles, since NA was immunohistochemically detected in both pea and tomato vacuoles, and its concentration was shown to increase when iron was supplied in excess (Pich et al., 2001). These findings suggest the requirement for NA or Fe-NA transporters on the vacuolar membrane, and YSL transporters are potential candidates to fulfill these roles (Haydon and Cobbett, 2007).

Chloroplasts are the major plant iron sink, as they require iron to carry out several metabolic processes such as photosynthetic electron transport, chlorophyll biosynthesis, Fe-S cluster assembly, and heme biosynthesis (Jeong and Guerinot, 2009). They also serve as important iron storehouses – up to 4500 iron atoms can be stored in a single ferritin protein, which is localized in plastids (Hintze and Theil, 2006). In fact, plastids contain ~80% of the iron found in leaf cells (Jeong and Guerinot, 2009). The reactive oxygen species (ROS) produced by the photosynthetic electron transport chain may cause

iron to undergo the Fenton reaction (Figure 4.1), thereby causing oxidative damage. Thus, levels of iron in the chloroplast must be tightly regulated, and excess must be either be bound to ferritin or to a carrier molecule such as nicotianamine, which was shown to hamper iron's ability to undergo the Fenton reaction (von Wiren et al., 1999).

Although the requirement for iron in the chloroplast has been clearly established, transporters responsible for iron transport into this organelle have not been fully elucidated. The permease PIC1 was shown to localize to the chloroplast inner envelope and was found to be critical for proper chloroplast development (Duy et al., 2007). PIC1 was also able to complement the yeast iron uptake mutant *fet3fet4*, as well as the copper uptake mutant *ctr1*, suggesting a metal transport function for this protein. However, metal transport was not shown *in planta*, and an alternative role for PIC1 in the translocation of proteins across the chloroplast inner envelope has been suggested (Teng et al., 2006).

Reduction of Fe by the chloroplast-localized reductase FRO7 also appears to be important for iron uptake into this organelle. *fro7* mutants displayed various photosynthetic defects and had reduced levels of Fe in their chloroplasts (Jeong et al., 2008). It was also previously shown that iron is efficiently transported across chloroplast inner envelope membranes as Fe^{2+} (Shingles et al., 2002). This is similar to the scenario at the root plasmalemma, in which Fe^{3+} must be reduced to Fe^{2+} via FRO2 prior to import via IRT1. However, *fro7* chloroplasts had a mere 33% reduction in the amount of iron in their chloroplasts, which seems to indicate that iron can still enter the chloroplast without the action of FRO7. This could indicate the presence of multiple chloroplast reductases,

or an alternative system of Fe import that does not require Fe to be reduced prior to transport.

Plant Strategies for Dealing with Iron Toxicity

As stated earlier, iron is essential for the plant, but it can also be toxic when it undergoes the Fenton reaction (Figure 4.1). The plant has two strategies for dealing with this – iron can either be sequestered, or it can be chelated to carrier molecules such as citrate or nicotianamine. Sequestration can occur either in the vacuole, or in the chloroplast where stored iron is bound by ferritin. In animals, ferritin is the primary storage form for Fe, although ferritins in both bacteria and *Chlamydomonas* have been shown to play important roles in iron detoxification (Carrondo, 2003; Busch et al., 2008; Long et al., 2008). Recent work in *Arabidopsis* seems to indicate that the major role of ferritin in the plant is not to store, but rather to detoxify by binding excess Fe and preventing oxidative damage (Ravet et al., 2009).

Another strategy to prevent toxicity involves the complexation of iron and other transition metals to carrier molecules. In plants, Fe is often found chelated to nicotianamine (NA), citrate, or phytosiderophores, the latter of which are produced only in the grasses (i.e. cereals, rice, maize) and are secreted into the rhizosphere to solubilize Fe^{3+} (Kim and Guerinot, 2007). However, all plants utilize both citrate and NA. Citrate is thought to be the main chelator of Fe in the xylem where the pH is weakly acidic (Hell and Stephan, 2003), and studies of the Arabidopsis mutant *frd3* (*man1*) have provided evidence for the role of citrate in long-distance Fe transport (Rogers and Guerinot, 2002;

Green and Rogers, 2004; Durrett et al., 2007). On a subcellular level, citrate is also found in vacuoles (Kramer et al., 2000), and is likely to be important for solubilization of metals in the acidic environment of the vacuolar lumen. However, a vacuolar citrate transporter has not yet been identified (Haydon and Cobbett, 2007).

The polyamine nicotianamine is a metal chelation molecule that is a key component of plant metal assimilation and homeostasis. NA is synthesized by all plants and plays a role in iron uptake, phloem transport, and cytoplasmic distribution, as well as ensuring iron solubility in the weakly alkaline environment of the plant cytoplasm (pH 7.3) (Douchkov et al., 2005; Weber et al., 2008). Additionally, NA plays a protective role by binding and converting iron to a non-Fenton active form (Curie et al., 2009). Nicotianamine synthase (NAS) genes are upregulated in plants grown under Zn, Cu, or Fe deficiency (Haydon and Cobbett, 2007), but paradoxically, an excess of nicotianamine causes plants to exhibit iron starvation responses (Cassin et al., 2009). In the case where this was studied, starvation responses appeared to be induced by excess NA accumulating in the apoplast, which was thought to be responsible for an increase in iron sequestration in this compartment (Cassin et al., 2009).

The overexpression of nicotianamine amino transferase (NAAT) in tobacco plants mimics the *chloronerva* mutant of tomato, because NAAT enzymatically consumes NA. In this overexpression line, iron provided alone was restricted to the main veins, but co-incubation with NA allowed for widespread distribution of iron in mesophyll cells (Takahashi et al., 2003). Citrate is also important in iron distribution. Under iron starvation, the concentration of citrate in the phloem increases (Schmidt, 1999). In

xylem, iron is transported as Fe-citrate complexes, since at pH 5.5 – 6 iron readily transfers from NA to citrate (Hell and Stephan, 2003). The importance of NA and citrate in the proper cellular distribution of iron is unquestionable, but transporters capable of moving these chelators or metal-chelator complexes are just recently beginning to be uncovered and characterized.

Transport of Iron Chelation Molecules

The maize transporter YS1 (yellow stripe 1) is known to transport Fe-NA and Ni-NA complexes (Roberts et al., 2004; Schaaf et al., 2004), so major candidates for the transport of Fe-NA complexes in *Arabidopsis* include members of the YSL (Yellow-Stripe Like) family of transporters, which belong to the oligopeptide transporter (OPT) family. The OPTs are rather poorly characterized, and are involved in the transport of tri-, tetra-, penta- and hexapeptides along with amino acid derivatives (Curie et al., 2009). There are eight YSLs in *Arabidopsis* (Curie et al., 2001). Loss-of-function insertional mutants of AtYSL1 accumulated more NA in shoots, but contained less iron and NA in seeds (Le Jean et al., 2005). AtYSL1 was expressed in the xylem parenchyma of leaves, and was upregulated in response to iron excess, consistent with long-distance circulation of iron and NA. AtYSL2 was downregulated under zinc and iron deficiency, however, it appeared unable to mediate transport of Fe-NA in Xenopus oocytes (Schaaf et al., 2005). AtYSL3 appears to act redundantly with AtYSL1, and double mutants are impaired in metal mobilization and exhibit iron deficiency symptoms, such as interveinal chlorosis (Waters et al., 2006).

The Arabidopsis FRD3 protein, which belongs to the multidrug and toxin efflux family, is hypothesized to be responsible for loading citrate into the xylem (Durrett et al., 2007). Xylem exudate from *frd3* plants contained less citrate and iron than wild type exudate, and supplementation of growth media with citrate was able to rescue *frd3* phenotypes. Additionally, FRD3 mediated transport of citrate in *Xenopus* oocytes. Another potential citrate transporter in Arabidopsis is AtIREG1. Although its biological function remains to be tested, AtIREG1 was hypothesized to be involved in vessel loading of iron (Curie and Briat, 2003), and was found to be downregulated in plants overexpressing DwMYB2, which are impaired in root-to-shoot iron translocation (Chen et al., 2006). It was postulated that the downregulation of AtIREG1 was likely to be the cause of the impaired iron translocation in this overexpression line. As previously mentioned, iron is transported as Fe-citrate in the xylem, therefore AtIREG1 may play a similar role to FRD3. FRD3 was also downregulated in DwMYB2 overexpressors, however, this downregulation was found in both roots and shoots, while AtIREG1 downregulation was found only in roots. *frd3* mutants have only a 40.2% reduction in their xylem citrate levels, thus, it is likely that there are other citrate efflux transporters that can compensate for the loss of FRD3 function (Durrett et al., 2007). AtIREG1 may be one of these transporters.

Intracellular Transport of Iron Chelation Molecules

Besides playing major roles in long distance iron transport, both citrate and NA are also known to be present in the intracellular environment. The plant must maintain low levels

of cytoplasmic iron to avoid toxic effects of iron accumulation (as discussed earlier), thus, plant vacuoles are likely to play a major role in storing excess iron and releasing it as needed. Evidence for this is found in pea mutants that overaccumulate iron – in these plants, NA concentrations in the vacuole rise, whereas under normal conditions, NA is found mostly in the cytoplasm (Pich et al., 2001). However, a vacuolar NA transporter has not yet been identified.

Citrate was thought to be transported into barley mesophyll vacuoles via malate transporters, with transport being ATP-dependent (Rentsch and Martinoia, 1991). However, recent results refute this data and maintain that citrate and malate are transported via separate transporters (Hurth et al., 2005). Citrate is likely to be the predominant ligand for zinc in *Thlaspi caerulescens* (Salt et al., 1999; Kupper et al., 2004), and the *Arabidopsis* protein ZIF1 (Zinc-Induced Facilitator1) has emerged as potential transporter for a Zn ligand. ZIF1 localizes to the vacuolar membrane, is induced in plants exposed to zinc, and belongs to the major facilitator superfamily (MFS), which transport a wide range of small organic molecules (Haydon and Cobbett, 2007).

Despite the essential need for iron and other metals in chloroplasts and mitochondria, it is unknown whether these metals exist in chelated form within these organelles. Using X-ray microanalysis in the NA-less tomato mutant *chloronerva*, electron-dense inclusions consisting of iron and phosphorus were detected in chloroplasts of palisade parenchyma (Becker et al., 1995). These inclusions disappeared after treatment with NA, indicating that NA may be acting in the chloroplast to keep iron

soluble. An independent study (Liu et al., 1998) also detected iron-containing deposits (using energy spectroscopic imaging, or ESI) in *chloronerva* chloroplast stroma, as well as in vacuoles and mitochondria. Application of NA led to a significant decrease of these deposits. These experiments seem to indicate that NA is required to maintain a soluble pool of iron within plant organelles, however, no organellar NA transporter has yet been identified.

Summary

Iron is an important micronutrient for plants because of the essential roles it plays in photosynthesis, respiration, and chlorophyll biosynthesis. However, levels of iron in the plant body must be tightly regulated, because when it accumulates in excess, iron generates cytotoxic hydroxyl radicals via the Fenton reaction (Jeong and Guerinot, 2009). Additionally, iron is not readily bioavailable due to its relative insolubility, especially at neutral or slightly basic pH. Iron chelators, such as citrate and nicotianamine, are important both for iron solubility and iron detoxification. Localization of iron to the proper compartment and maintenance of accessible iron stores are crucial for maintaining iron homeostasis. Plant methods of iron acquisition from the environment have been well studied, but transporters involved in plant organellar iron transport are only recently beginning to be uncovered. Additionally, much remains to be learned about the role of chelators in intracellular compartments, especially chloroplasts and mitochondria.

CHAPTER V

Preliminary Studies to Uncover the Natural Function of MAR1

Results and Discussion

It is unlikely that evolutionary pressures would have selected for a means of entry for toxic antibiotics into plant chloroplasts. Therefore, we propose that *MAR1* has a more "conventional" role in the plant, and the transport of antibiotics is an opportunistic effect. In fact, there are several transport proteins in bacteria that are capable of moving both antibiotics and metabolites, such as the mexAB/oprM multidrug efflux operon of *P. aeruginosa*, which is also involved in the secretion of the iron chelator, pyroverdine (Poole et al., 1993; Poole et al., 1993; Paulsen et al., 1996), and the Blt drug transporter of *B. subtilis*, which also transports polyamines (Neyfakh et al., 1991; Jack et al., 2001). Given its sequence similarity to ferroportin, it is possible that *MAR1* could be involved in iron transport.

MAR1 is Unable to Complement a Yeast Strain Defective in Iron Uptake

The expression pattern of *MAR1* does not yield many clues as to its potential function. A promoter-reporter fusion using *pMAR1::GUS* transgenic plants demonstrated that *MAR1* is expressed throughout the plant body in young seedlings (Figure 5.1 A and B). In addition, *MAR1* appears to be fairly evenly expressed in most tissue types based on

AtGenExpress data (Figure 5.1 C). In an attempt to test the possibility that MAR1 is involved in iron transport, we expressed *MAR1* cDNA in the yeast double mutant *fet3fet4*, which is defective in low and high affinity iron uptake across the plasma membrane and therefore unable to grow on media without iron supplementation (Dix et al., 1994). *Arabidopsis* iron transporters, such as PIC1 and IRT1, have been shown to complement this strain, allowing for growth either with minimal supplementation (for PIC1, 10 μM FeCl3) or without supplementation (for IRT1) (Eide et al., 1996; Duy et al., 2007). We found that *MAR1* expressed in vector pVV214 was unable to complement *fet3fet4* on SD media ± 5, 10, or 20 μM $FeCl_3$ (data not shown). The addition of 50 μM Fe-citrate to the media restored growth of all strains, with or without *MAR1* expression, as expected (Roberts et al., 2004). Our negative results here are probably unsurprising, given MAR1's localization to the yeast mitochondria (Figure 3.4), which the typical localization for chloroplast membrane proteins expressed in yeast (Versaw and Harrison, 2002; Jeong et al., 2008).

35S::*MAR1* Plants Display a Phenotype of Chlorosis

We observed no phenotypic differences between 35S::MAR1, *mar1*, and corresponding wild type plants when grown for two weeks under limiting iron conditions (either 100 μM or 1 mM ferrozine; data not shown), or when grown for two weeks under conditions of iron toxicity (500, 550, and 600 μM Fe-EDTA). Interestingly however, we observed a visible and quantifiable phenotype of chlorosis in 35S::*MAR1* seedlings when grown for

two weeks on plain MS media (Figure 5.2 B) or MS media supplemented with 50 μM Fe-EDTA (Figure 5.2 A, first plate). Chlorosis is a common symptom of iron deficiency in plants, since iron is essential for chlorophyll biosynthesis (Vert et al., 2002). When 35S::*MAR1* plants were grown in soil, they also appeared slightly more chlorotic than wild type, both in leaves and stems. Chlorosis was especially prominent along the midvein and older areas of cauline leaves, which also displayed a slightly more pointed tip morphology compared to L*er* (Figure 5.2 D). Chlorosis of plate-grown seedlings persisted until media was supplemented with 300 μM Fe-EDTA (Figure 5.2 A and C). Our results here suggest that the overexpression of *MAR1* creates an iron-limiting condition for the plant.

MAR1 is Downregulated Under Iron Limiting Conditions

One of the *MAR1* homologs in *Arabidopsis*, *AtIREG2*, was found to be upregulated under iron deficiency (Schaaf et al., 2006). With this in mind, we examined *MAR1* for transcriptional changes under iron limitation and iron excess. Plants were grown in liquid culture for 14 days, and baseline tissue samples were taken before addition of either 600 μM Fe-EDTA (iron excess) or 300 μM ferrozine (iron limitation). We observed a 60% decrease in *MAR1* expression after 4 days of growth under iron deficiency (Figure 5.3 A), along with the expected upregulation of *IRT1* under similar conditions (Stacey et al., 2008). We also observed a downregulation of *MAR1* when plants were grown for two weeks on plates containing a lower concentration of ferrozine (100 μM; Figure 5.3 B). A

subsequent increase in *MAR1* transcription was not observed when iron levels were elevated for four days (Figure 5.3 C), despite the expected downregulation of *IRT1* under these conditions (Figure 5.3 C). Because the chlorosis of 35S::*MAR1* can be rescued by excess iron, and *MAR1* is downregulated under limiting iron conditions, we postulate that *MAR1* may play a role in iron chelation, storage, or sequestration.

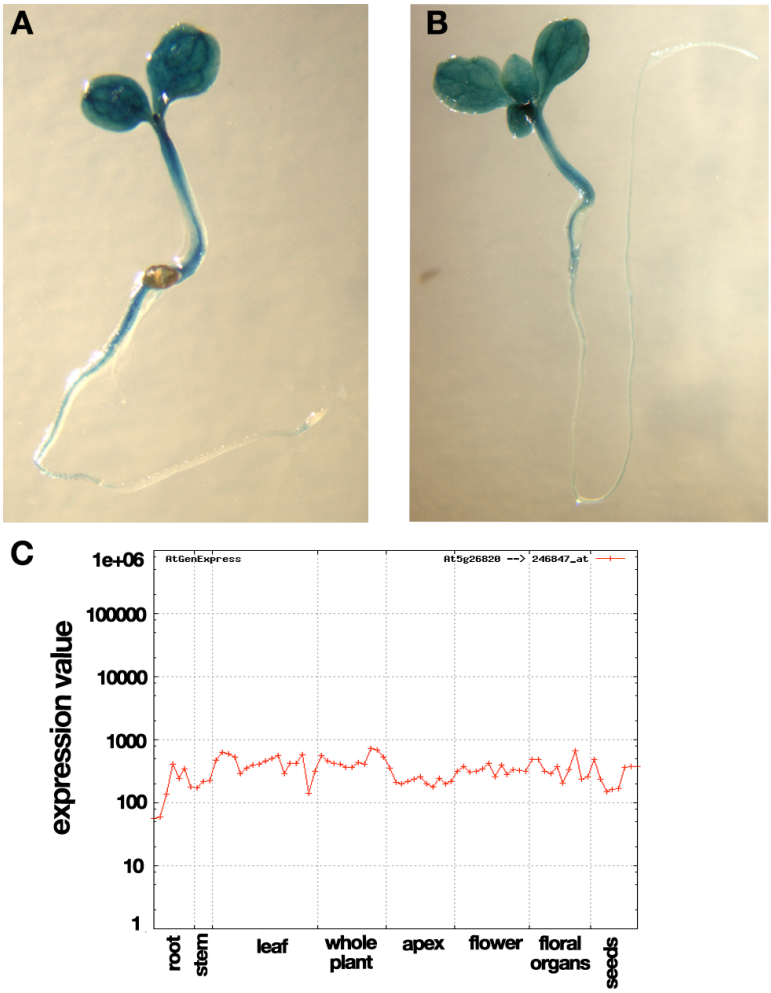

Figure 5.1. ***MAR1*** **Expression Pattern.** (A and B) *MAR1* is strongly expressed in all tissues of young seedlings. The GUS gene was expressed under control of the *MAR1* promoter (888 bp upstream of the *MAR1* coding sequence). Histochemical staining was performed on several independent lines using X-GLUC. (A) Representative 7-day-old seedling. (B) Representative 14 day-old seedling. (C) Developmental expression of *MAR1*. mRNA levels for *MAR1* (At5g26820) were obtained from AtGenExpress (Schmid et al., 2005).

MAR1 May Sequester Iron in the Chloroplast

The chlorosis phenotype of the *MAR1* overexpression line gives insight into the natural function of the MAR1 protein. Since this phenotype is rescued by iron feeding (Figure 5.2 A and C), MAR1 may play a role in the chelation, storage, and/or sequestration of iron. If so, we might expect a decrease of MAR1 transcript under iron limiting conditions, which is what was observed (Figure 5.3 A and B). Under limiting conditions, we also saw the expected increase in the transcript of the major root iron transporter *IRT1* (Eide et al., 1996; Korshunova et al., 1999; Rogers et al., 2000). *IRT1* is highly upregulated under iron limitation to increase the supply of iron to the plant cell (Connolly et al., 2002). This upregulation leads to an increase in cytoplasmic iron, but due to the poor substrate specificity of *IRT1*, it also results in increasing cytoplasmic levels of other toxic divalent metal cations, such as nickel. One of the *MAR1* homologs in *Arabidopsis*, *AtIREG2*, is proposed to play a role in the vacuolar sequestration of excess nickel accumulated under iron-limiting conditions, due to the action of *IRT1* (Schaaf et al., 2006). Schaaf *et al.* showed that *AtIREG2* was upregulated under iron deficiency, in contrast to *MAR1*, which is downregulated (Figure 5.3 A and B). Thus, we suggest that MAR1 and AtIREG2 play distinct roles in the plant cell. Their expression patterns are quite different – *AtIREG2* is mainly expressed in the root (Schaaf et al., 2006), while *MAR1* is highly expressed in all tissues (Figure 5.1 C). AtIREG2 localizes to the vacuole (Schaaf et al., 2006), while MAR1 localizes to the chloroplast (Figure 3.1 and 3.2). Despite these differences, we hypothesize that MAR1 and AtIREG2 both act to sequester

metal – AtIREG2 sequesters nickel in the vacuole, while MAR1 may be sequestering iron in the chloroplast.

As mentioned previously, *AtIREG1* was postulated to be involved in vessel loading of iron (Curie and Briat, 2003), and its downregulation in DwMYB2 overexpressors (Chen et al., 2006) may be the cause of the disruption in iron translocation (from root to shoot) observed in these plants. Because citrate appears to be the major chelator for iron in the xylem (Haydon and Cobbett, 2007), it is possible that *AtIREG1* exports citrate (or an iron-citrate conjugate) from root cells into the vasculature, playing a role similar to *FRD3*, which mediates citrate efflux into root vasculature (Durrett et al., 2007). With this in mind, we postulate that MAR1 may also be acting to transport an iron chelator, such as citrate or nicotianamine. Since cytosolic iron homeostasis depends on NA (Hell and Stephan, 2003), and NA appears to be present in chloroplasts (Becker et al., 1995; Stephan, 1995), it may be possible that *MAR1* transports NA into the chloroplast, where it likely is required to maintain iron solubility in the weakly alkaline environment of the stroma (Wu and Berkowitz, 1992).

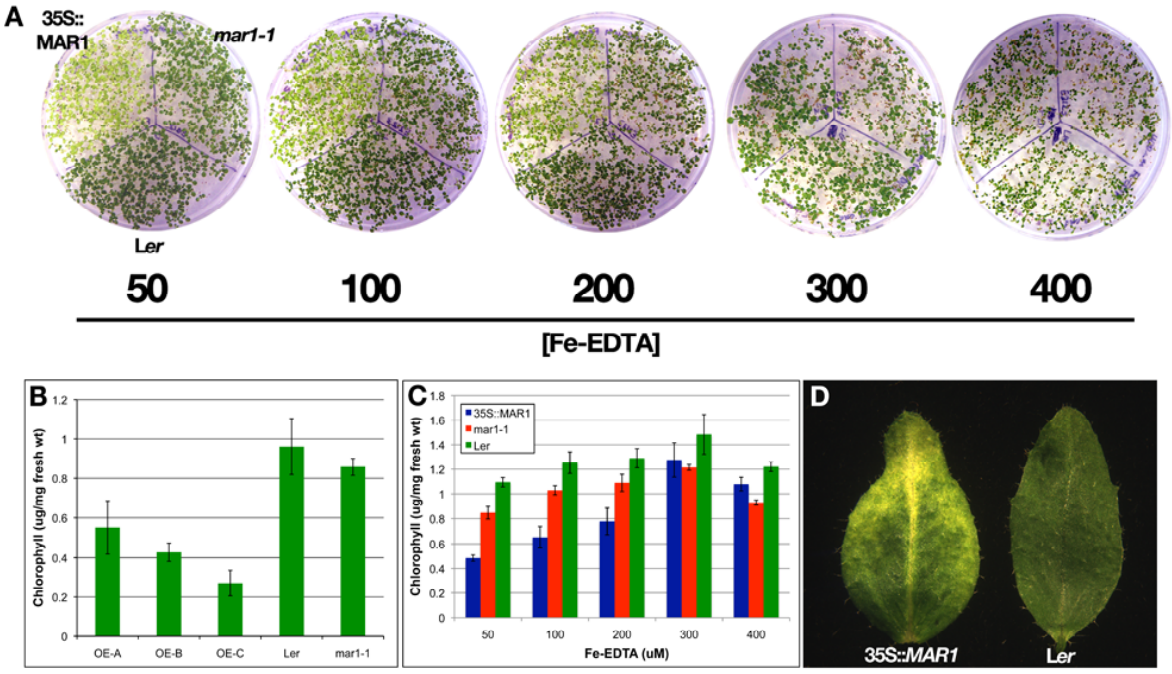

Figure 5.2. Chlorosis of 35S::*MAR1* is Rescued by 300 µM Fe-EDTA. (A) Plants were grown for two weeks on varying concentrations of Fe-EDTA (µM as indicated) before photographing. For each plate, the upper left section contains 35S::*MAR1* seedlings, the upper right contains *mar1-1* seedlings, and the lower section contains L*er* wild-type seedlings. (B) Chlorophyll content of three *MAR1* overexpression lines (OE-A, OE-B, and OE-C), L*er* and *mar1-1* grown on MS plates supplemented with 1% sucrose for two weeks (as described in materials and methods). Each bar represents the average chlorophyll content of three batches of seedlings (±SD) (C) Chlorophyll content of 35S::*MAR1*, *mar1-1*, and L*er* seedlings after two weeks growth on MS supplemented with varying concentrations of Fe-EDTA (as indicated). Chlorophyll was extracted and quantified as in (B). (D) Chlorosis phenotype of 35S::*MAR1* leaves from plants grown in soil for 32 days.

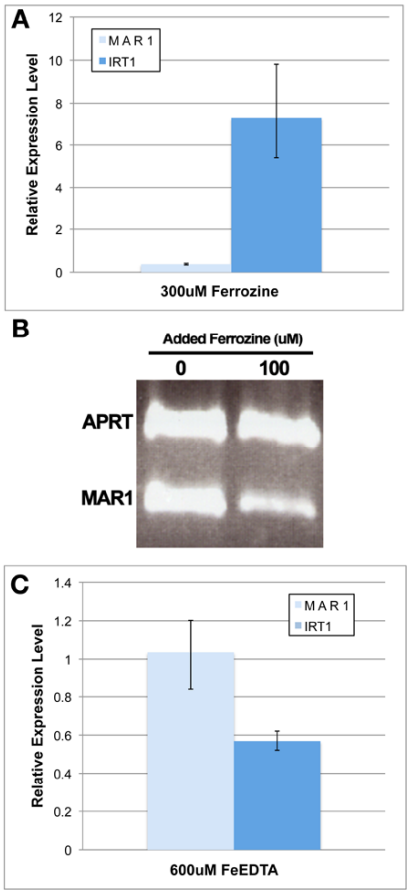

Figure 5.3. ***MAR1*** **is Downregulated Under Iron Deficiency.** (A) Plants were grown for two weeks in liquid MS supplemented with 1% sucrose, and seedling tissue samples were taken before and after four days of incubation in 300 μM ferrozine. Expression levels of *IRT1* and *MAR1* are expressed as fold-changes relative to their expression prior to ferrozine treatment. Error bars denote range of expression. (B) Plants were grown for two weeks on media containing 100 μM ferrozine prior to RNA extraction and RT-PCR. Equal amounts of each reaction were loaded on an agarose gel, and APRT was included as an internal control. (C) Plants were grown exactly as in (A), except that 600 μM of Fe-EDTA was added on day 14 (instead of ferrozine). Expression levels of *IRT1* and *MAR1* are expressed as fold-changes relative to their expression prior to Fe-EDTA treatment. Error bars denote range of expression.

The chlorosis phenotype of 35S::*MAR1* plants could be due to excess NA accumulating in the chloroplast, where it may sequester iron, creating the phenotype of iron deficiency. The phenotype observed in leaves of mature 35S::*MAR1* plants is the opposite of that seen in plants lacking NA (such as *chloronerva*) – instead of interveinal chlorosis in young tissues, chlorosis arises in the midvein and in older tissues. This unusual chlorosis pattern may be the result of a re-distribution of the cytoplasmic NA pool to the chloroplast. This has the dual effect of restricting NA from performing its role in phloem transport of iron and other metals (von Wiren et al., 1999), and also sequestering iron itself, thus preventing it from being re-distributed throughout the plant body. Iron, applied in excess (300 μM), is able to rescue the chlorosis phenotype, and the *MAR1* gene is downregulated under iron deficiency to prevent sequestration of needed iron. It may be that there is no increase in MAR1 expression under iron excess (600 μm) due to the negative effects of NA over-accumulation (Cassin et al., 2009). It is well known that aminoglycosides mimic polyamines, and can use their inward transport systems for entering both bacteria and eukaryotic cells (Van Bambeke et al., 2000). Since NA is a polyamine (Ling et al., 1999), it may be a good potential candidate for a natural substrate of MAR1. This hypothesis will require further investigation.

Since MAR1 is classified as a ferroportin, the possibility also remains that MAR1 is transporting iron, and the chlorosis seen in MAR1 overexpressors is a result of oxidative damage caused by excess iron accumulation in the chloroplast. If this is the case, chlorosis of overexpressors is relieved in the presence of high levels of exogenous

iron (300 μM) because at this level, the plant is likely to activate its many defense mechanisms against iron toxicity, such as downregulation of IRT1 and AtNRAMP3 (Vert et al., 2002; Ravet et al., 2009), upregulation of ferritin (Gaymard et al., 1996), increasing NA production (Pich et al., 2001), and activation of responses against oxidative stress (Fourcroy et al., 2004). If MAR1 does act to transport iron into the chloroplast, it may be regulated like AtSBL, a putative transporter hypothesized to import iron into chloroplasts for storage in ferritins (Wintz et al., 2003).

Both the chloroplast and mitochondria require metalloproteins for photosynthesis and respiration, respectively, though the question of how iron and other metals are allocated between the two organelles has not yet been addressed (Merchant et al., 2006). Since most photosynthetic components are down-regulated under iron limitation (Tognetti et al., 2007), one possibility is that under limiting conditions, iron is preferentially allocated to the mitochondria to maintain respiration. If this is the case, and MAR1 is acting to transport iron, we would expect to see a decrease in its expression under iron limitation, which is what was observed (Fig. 9A and B).

Chapter VI

Summary and Conclusions

In this study, we have uncovered, cloned, and characterized an interesting transporter protein from *Arabidopsis thaliana* that appears to be a means by which aminoglycoside antibiotics can opportunistically gain entry to their intracellular targets. We have isolated several mutations in the locus *Multiple Antibiotic Resistance* (*MAR1*) that are capable of conferring resistance to multiple aminoglycoside antibiotics (Figure 2.1 A, B and C). This resistance does not extend to antibiotics of other classes, even to the structurally similar aminocylitol, spectinomycin (Figure 2.1 D, Figure 2.2). Overexpression of the *MAR1* locus causes hypersensitivity to multiple aminoglycoside antibiotics, both in wild type and *mar1* mutant backgrounds (Figure 2.9 and Figure 2.10). Additionally, yeast expressing *MAR1* are hypersensitive to the aminoglycoside, G418 (Figure 3.3). *MAR1* encodes a protein with 11 putative transmembrane domains with low similarity to ferroportin1 from *Danio rerio*. A MAR1::YFP fusion protein localizes to the chloroplast (Figure 3.1 and 3.2), and chloroplasts from plants overexpressing MAR1 accumulate more of the aminoglycoside, gentamicin, while *mar1-1* mutant chloroplasts accumulate less than wild type (Figure 3.6).

Multiple drug and antibiotic resistance is often conferred by gain of function mutations in ATP-driven multidrug exporters with little substrate specificity. However, greater knowledge about import mechanisms must be achieved in order to truly

understand and overcome resistance, especially for hydrophilic antibiotics such as the aminoglycosides, which do not readily diffuse across membranes. *mar1* represents an interesting example of plant antibiotic resistance that is based on the restriction of antibiotic entry into a subcellular compartment. Knowledge about this process – and other processes of antibiotic entry – could enable the production of crop plants that are incapable of antibiotic accumulation, aid in development of phytoremediation strategies for decontamination of water and soils polluted with antibiotics, and further the development of new plant-based molecular markers. The work described here also contributes to our understanding of how plants interact with the antibiotics they encounter, both in the laboratory (where aminoglycosides such as kanamycin are used heavily to select for transgenics) and in the natural environment.

It is unlikely that evolutionary pressures would have selected for a means of entry for toxic antibiotics into plant chloroplasts. Therefore, *MAR1* must have a more "conventional" role in the plant, and the transport of antibiotics is an opportunistic effect. Given the similarity of MAR1 to ferroportin from *Danio rerio* and iron-regulated transporters in *Arabidopsis*, it is likely that MAR1 plays a role in iron transport and/or homeostasis. We have found that *MAR1* overexpression lines are slightly chlorotic, and chlorosis is rescued by exogenous iron (Figure 5.2). *MAR1* expression is also downregulated by low iron (Figure 5.3). These data suggest that MAR1 is likely to be involved in cellular iron homeostasis.

It is well known that aminoglycosides mimic polyamines, and can use their inward transport systems for entering both bacteria and eukaryotic cells (Van Bambeke et al., 2000). Since NA is a polyamine (Ling et al., 1999), it may be a good potential candidate for a natural substrate of MAR1. This hypothesis will require further investigation. Since MAR1 is classified as a ferroportin, the possibility also remains that MAR1 is transporting iron, and the chlorosis seen in MAR1 overexpressors is a result of oxidative damage caused by excess iron accumulation in the chloroplast. Regardless of whether MAR1 transports iron, an iron chelator, or an iron-chelator conjugate, knowledge about its function in this area will contribute much to the field of metal transport and homeostasis, which is particularly lacking in data regarding chloroplast iron transport.

Chapter VII

Materials and Methods

Plant Materials and Growth Conditions. The original antibiotic resistant mutant, line E2-123 (*mar1-1*), was generated via EMS mutagenesis of line E2-6 (L*er* background, antibiotic sensitive) (Kilby et al., 1992). The E2-6 line contains a methylation silenced *nptII* gene at an unknown location. The original mutagenesis and screen was performed to try to identify components of the DNA methylation pathway. However, the T-DNA containing the silenced *nptII* gene segregates away from the *MAR1* locus. The original E2-123 (*mar1-1*) line was outcrossed to wild type L*er* one time, and one subsequent homozygous *mar1-1* F2 progeny, without any T-DNA (verified by Southern blot and PCR analysis), was used as the parent in the experiments presented here.

Plants were grown either in a growth room at 21°C, ambient humidity, under constant fluorescent illumination, or on Petri dishes in a Percival chamber under similar conditions.

Plant Transformation. All constructs to be used in plant transformation experiments were transferred to *Agrobacterium tumefaciens* GV3101 via electroporation. *Arabidopsis thaliana* plants were transformed by *Agrobacterium*-mediated transformation using the floral dip method (Clough and Bent, 1998). Primary transformants were selected in soil

or on MS plates using the herbicide Basta (1.5 µl/mL; AgrEVO, Germany). The progeny of at least three selfed, primary transformants were used for experiments.

Map-Based Cloning of *MAR1*. A total of 608 kanamycin-resistant F2 progeny from a cross of the *mar1-1* mutant (F2 minus T-DNA as described above) to Col, and the *mar1-1* and Col parents were genotyped using microsatellite loci polymorphic between Col and L*er*. The *mar1-1* mutation was initially mapped to an interval between microsatellite markers *nga139* and *PHYC* on Chromosome V (www.arabidopsis.org). Additional polymorphic microsatellite markers were generated using microsatellite-finding software (Microsat Radar) and testing for length polymorphisms in parental lines. For finer resolution mapping, short intervals evenly spaced in the narrowed region were sequenced to find SNPs and INDELs. Markers defining a final mapping interval of 150 kB (33 genes) were at 9.34 mB and 9.48 mB on Chromosome V, both of which are SNPs between Col-0 and L*er*.

Resistant seedlings were selected after two weeks of growth on MS media plus kanamycin (25mg/L). Genotype data were analyzed using MetaPhor® agarose gels (Cambrex, Rockland ME) and by fragment analysis using the Applied Biosystems 3730 Genetic Analyzer and GeneMapper® software.

Gene Cloning and Plasmid Construction. All clonings were done using the Gateway™ system (Invitrogen). All *attB*-tailed PCR products were initially cloned into

pDONR222 using BP Clonase, and sequence verified before subcloning into various plant expression vectors (using LR Clonase) mentioned below.

35S::*MAR1*

The *MAR1* locus (At5g26820) was amplified by PCR (TripleMaster PCR system; Eppendorf) from L*er* (wt) genomic DNA using *attB*-tailed gene specific primers (Table 7.1). *MAR1* was then subcloned into the plant overexpression vector pB7WG2 (Karimi et al., 2002) for subseqent *Agrobacterium*-mediated transformation of Col-0, L*er*, E2-6, and *mar1-1* plants. At least three independent Basta resistant transformed lines were isolated for analysis for each vector-genotype combination.

35S::*MAR1-YFP* and 35S::*YFP-MAR1*

MAR1 cDNA in vector pENTR/SD-DTOPO was obtained from the ABRC stock center (www.arabidopsis.org, clone name: U16896). *MAR1* cDNA was amplified by PCR (Accuprime HiFidelity; Invitrogen) from this vector using specific primers (Table 7.1). *MAR1* cDNA lacking a stop codon was subcloned into vector pH7YWG2 (Karimi et al., 2005) in frame to *YFP* for subsequent expression in *Arabidopsis* Col-0 protoplasts and *mar1-2* plants. N-terminal fusions (35S::*YFP-MAR1*) were constructed exactly as above, except that *MAR1* cDNA was cloned into the vector pB7WGY2 (in frame with YFP) and the stop codon was retained.

35S::*MAR1tp-YFP*

The first 162 nucleotides of *MAR1* were amplified by PCR (Accuprime HiFidelity; Invitrogen) using specific primers (Table 7.1). Vector pH7YWG2 (Karimi et al., 2005) was used for subsequent expression in *Arabidopsis* Col-0 protoplasts.

MAR1 promoter::GUS

The 888 bp *MAR1* promoter region was amplified by PCR (TripleMaster PCR system, Eppendorf) from L*er* genomic DNA using *attB*-tailed primers (Table 7.1). Vector pBGWFS7 (Karimi et al., 2002) was used for subsequent *Agrobacterium*-mediated transformation of plants. Six independent Basta resistant transformants were allowed to self, and progeny seed was germinated on MS media and grown for 7 or 14 days prior to analysis.

***Arabidopsis* Protoplast Transformation.** Protoplasts were isolated from 20 day-old seedlings and transformed according to methods previously described (Weigel and Glazebrook, 2002). Constructs used for transformation included *35S::MAR1-YFP* (described above), 35S::*MAR1tp-YFP* (described above), and 35S::*YFP* (pCL-eYFP-FL; a gift from Dr. Enamul Huq). Transformed protoplasts were allowed to incubate overnight under continuous light at 22°C prior to confocal microscopy.

35S::*MAR1*	FW – 5'-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTG **ATG GTT GTT TCA ATG GCT TTG GTC A** REV – 5'-GGGG AC CAC TTT GTA CAA GAA AGC TGG GT**TCA ATT TGA GAG AGG GTC GAA GGA G**
35S::*MAR1-YFP*	FW – same as 35S::*MAR1* FW REV – 5'-GGGG AC CAC TTT GTA CAA GAA AGC TGG GT**G ATT TGA GAG AGG GTC GAA GGA G**
35S::*MAR1tp-YFP*	FW – same as 35S::*MAR1* FW REV – 5'-GGGG AC CAC TTT GTA CAA GAA AGC TGG GT**C GCG AGA ACA TGA AGG TGA AGA**
MAR1::*GUS*	FW – 5'-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTG **ATA AAT CAC TTT CTC TTT TG** REV – 5'- GGGG AC CAC TTT GTA CAA GAA AGC TGG GT **GAG GAG AAT CAA TTT ATA GA**
Primers for RT-PCR:	
IRT1	FW – GAGTCATTGCCATGGTCTTGGA REV – GTATACTCAGCCTGGAGGATACAACCG
MAR1	FW – GGTCAAACGAGTTGGCATTT REV – GAGACAGAACCGCGAGAAAC
ACTIN	FW – TCCATTCTTGCTTCCCTCAG REV – ATCATACTCGGCCTTGGAGA
APRT	FW – ATGGCGACTGAAGATGTGCAA REV – TTAAGCAGCCGACTTTACAAG

Table 7.1. Primer Sequences. Gene-specific sequences of Gateway primers are in bold

Confocal Microscopy Analysis of 35S::MAR1-YFP, 35S::MAR1tp-YFP, and 35S::YFP. A Leica SP2 AOBS confocal laser scanning microscope was used for visualizing fluorescence images from *Arabidopsis* protoplasts and leaves. Excitation was at 514 nm and the emission signal was collected between 525 nm and 590 nm for YFP fluorescence, and between 622 nm and 700 nm for chlorophyll autofluorescence. Untransformed protoplasts and leaves were also examined as controls.

T-DNA Knockout Lines *mar1-2* and *mar1-3*. T-DNA insertion alleles were identified from the SIGnAL (Salk Insititute Genomic Analysis Laboratory) collection. *mar1-2* carries a T-DNA insertion in the 11th exon of At5g26820 (Salk_034189, position 9436545 on chromosome V). *mar1-3* carries a T-DNA insertion in the 9th exon of At5g26820 (Salk_009286, position 9436095 on chromosome V). Lines were confirmed homozygous by PCR and by segregation analysis on kanamycin.

Gene expression analysis of *MAR1* overexpression and T-DNA lines. Seedlings from 35S::*MAR1* (two independent lines, F2D and F2Y), *mar1-2, mar1-3*, and L*er* (wild-type) were grown on MS media containing 1% sucrose at 21°C under continuous white light in a Percival growth chamber. Total RNA was extracted from two-week-old seedlings using the QIAgen RNeasy Plant mini kit with on-column DNAse treatment. RNA (2 μg) was used in 20 μl reverse transcription reactions containing 250 nM actin and *MAR1* gene-specific reverse primers. For each target (actin and *MAR1*), five PCR reactions

containing 400 nM primers and 1 μl first strand cDNA as a template were performed using SYBR® green master mix (ABI) and a spectrofluorometric thermal cycler (ABI 7900HT). The comparative cycle threshold method was used to analyze the results (User Bulletin 2, ABI PRISM Sequence Detection System).

Gene expression analysis of *MAR1* under varying iron conditions. Seeds from L*er* (wild-type) were grown in Erlenmeyer flasks containing 200 mL liquid MS media supplemented with 1% sucrose at 21°C under continuous white light on a shaker set to constant RPM in a Percival growth chamber. After 14 days of growth, several whole seedlings (roots and shoots) were removed, and RNA was extracted using the QIAgen RNeasy Plant mini kit with on-column DNAse treatment. Media was then supplemented with either 600 μM Fe-EDTA (iron excess) or 300 μM ferrozine (iron restriction), and remaining seedlings were allowed to incubate for a further 4 days. On day 4, RNA was extracted from remaining whole seedlings as above.

RNA (4 μg) from each sample was used in 40 μl reverse transcription reactions containing 250 nM actin, *IRT1*, and *MAR1* gene-specific reverse primers. For each target (actin, *IRT1* and *MAR1*), five PCR reactions containing 400 nM primers and 2 μl first strand cDNA as a template were performed using SYBR® green master mix (ABI) and a spectrofluorometric thermal cycler (ABI 7900HT). The comparative cycle threshold method was used to analyze the results (User Bulletin 2, ABI PRISM Sequence Detection System).

For gel-based RT-PCR, plants were grown for two weeks on plates containing 100μM ferrozine. On day 14, whole seedling tissue (root and shoot) was harvested and RNA extracted as above. 2μg of RNA was used as a template for each cDNA reaction (containing both *MAR1* and *APRT* primers), and equal amounts of cDNA reactions were loaded on a gel. Products were visualized with UV and ethidium bromide.

Quantification of Antibiotic Resistance in Plants. Titrations of the antibiotics kanamycin, gentamicin, streptomycin, tobramycin, amikacin, and apramycin were established to determine the concentration at which the greatest difference in resistance could be observed between wild type and mutant *mar1-1* when plated on MS media plus antibiotic. These concentrations were determined to be kanamycin 25 mg/L, gentamicin 70 mg/L, streptomycin 75 mg/L, tobramycin 40 mg/L, amikacin 100 mg/L, and apramycin 200 mg/L. Increased resistance was not found to hygromycin (1-10 μg/mL, 15 μg/mL, 20 μg/mL were tested), Tetracycline (2.5 μg/mL, 5 μg/mL, 10 μg/mL), Chloramphenicol (2.5 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL, 30 μg/mL), Erythromycin (5 μg/mL, 10 μg/mL, 15 μg/mL, 20 μg/mL, 25 μg/mL, 35 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL), Tylosin (4 μg/mL, 8 μg/mL, 16 μg/mL), Paromomycin (25 μg/mL, 50 μg/mL, 100 μg/mL), G418 (25 μg/mL, 50 μg/mL, 100 μg/mL, 300 μg/mL, 500 μg/mL) (data not shown).

Seeds of mutant lines *mar1-1* and *mar1-2* along with a corresponding wild type line (Col x L*er*, F4) and an unrelated kanamycin resistant T-DNA insertion line

(Salk_030942, which interrupts *MYB5*) were surface sterilized and plated onto MS media and MS plus antibiotic. After 48 hrs vernalization, plates were moved to a 22°C incubator under constant light conditions for 14 days. Chlorophyll was extracted and quantified in triplicate according to methods described previously (Porra et al., 1989).

Yeast Transformation and Antibiotic Susceptibility Assays. *MAR1* and *mar1-1* cDNAs were cloned into vector pVV14 (Van Mullem et al., 2003) via the Gateway™ method, and the *Saccharomyces cerevisiae* strain BY4700 (*MAT***a** *ura3*Δ*0*) was transformed with these vectors using standard methods (Elble, 1992). BY4700 transformed with pVV214 alone served as a control. Eight individual clones from each line were selected from -URA dropout plates and PCR checked for presence of the transgene. Of the positive clones, three were selected and grown overnight at 30°C in 5 mL of -URA liquid dropout media. Cultures were then standardized to 0.01 OD (λ600) before addition of various concentrations of G418 or cycloheximide (Figure 3.3). After 48 hours of growth at 30°C, OD was recorded for each culture. The antibiotic susceptibility experiment was carried out in triplicate.

MAR1 Localization in Yeast. *MAR1* cDNA (with stop codon removed) was cloned into vector pAG426GPD-ccdB-EGFP (Addgene plasmid 14204) via the Gateway™ method, and the yeast strain BY4700 was transformed as described above. pAG426GPD-ccdB-EGFP alone was used as a control. A mixed population of transformed and

untransformed cells was incubated in a 500 nM solution of MitoTracker Red CMXRos (Invitrogen) for 20 minutes at room temperature. A Leica SP2 AOBS confocal laser scanning microscope was used for visualizing fluorescence images. Excitation was at 514 nm, and the emission signal was collected between 525 nm and 540 nm for GFP fluorescence, and between 600 nm and 650 nm for MitoTracker Red.

Chloroplast isolation and antibiotic uptake assays. Intact chloroplasts were isolated basically according to the method of Weigel and Glazebrook (Weigel and Glazebrook, 2002), with the following modifications based on the method of Aronsson and Jarvis to ensure that chloroplasts were import-competent (Aronsson and Jarvis, 2002): Sodium ascorbate was added to the XPL buffer to a final concentration of 50 mM (instead of 5 mM), seedlings were grown for 14-16 days on MS media (instead of in soil), 7-10 g of tissue was harvested, and plant tissue was ground with 50 mL XPL using a mortar and pestle until the XPL turned slightly green. The mixture was filtered through two layers of miracloth, and remaining plant material was returned to the mortar, ground with 20 mL XPL, and re-filtered. This 20 mL grinding step was then repeated 2 additional times. After gradient centrifugation, chloroplasts collected from the gradient interface were washed with 10 mL HMS buffer (50 mM HEPES-KOH pH 8, 3 mM MgSO4, 0.3 M sorbitol) and resuspended in 2 mL HMS. Chloroplasts were consistently determined to be >80% intact based on photoreduction of ferricyanide (Sigma Chloroplast Isolation Kit Technical Bulletin, 2002).

Chloroplasts were counted using a hemocytometer, and a standard number was used for each reaction (Figure 3.6 legend). The uptake reaction buffer was HMS + 10 mM carbonate + 0.2% w/v BSA. Gentamicin was added to a final concentration of 12.5 mg/mL, and uptake reactions were carried out on a rotator in a Percival chamber under constant fluorescent illumination for given time periods (Fig. 3.6 A). Negative controls were incubated in HMS uptake buffer without gentamicin. To stop the uptake reaction, tubes were spun at 1000 x g for 2 minutes in a microcentrifuge, supernatant was decanted, and chloroplasts were washed with 500 μl HMS buffer. This was repeated for a total of three washes. Chloroplasts were then incubated in 150 μl CP lysis buffer (20 mM HEPES pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 10 mM DTT, 10% v/v glycerol, and 1% w/v PVP) on ice for one hour with occasional vortex. Supernatants were collected after centrifugation (3000 x g for 5 minutes) and stored at -20°C until use in dot blot.

Dot blots for antibiotic detection in chloroplast lysates were performed as follows: 2 μl of each lysate was spotted onto nitrocellulose membrane (pore size 0.2 μM) in triplicate (Figure 3.6 C), along with 2 μl of each of a set of standard gentamicin solutions (in CP lysis buffer) as positive controls (Figure 3.6 B). Spotted membranes were allowed to dry for 45 minutes before blocking with 1X PBS pH 7.4 + 0.05% v/v Tween20 + 5% w/v nonfat dry milk. Blocking time was 1 hour on a rotary shaker at RT. After the block, mouse anti-gentamicin antibody (AbCam, Cambridge MA) was applied (in blocking solution) at 1:1000 dilution, and incubation was carried out at 4°C overnight.

The membrane was then washed 2X for 15 mins each with PBS, 3X for 15 mins each with PBS + 0.05% v/v Tween 20, and 1X for 15 mins with PBS.

Goat anti-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnology) was applied (in blocking solution) at a dilution of 1:5000 and allowed to incubate for 1.5 hours on a rotary shaker at RT. The above washes were then repeated. The membrane was allowed to incubate for 1 minute in Western Lighting™ Plus-ECL solution (Perkin Elmer) before exposure to film (Kodak BIOMAX Light) for 10 sec to 1 minute. Images of developed film were analyzed using ImageJ64 (NIH). The image was inverted, and background was subtracted using a rolling ball radius between 60-80 pixels, depending on the blot (rolling ball radius should be equivalent to the size of the largest dot on the blot). The integrated density function was then used to measure the intensity of each dot. The average of three replicate dots (±SD) was graphed (Figure 3.6 A).

Whole seedling uptake. Approximately 2000 seeds were sterilized for each line and vernalized for 2 days at 4°C in 100 mL volumes of liquid MS growth media. Flasks were then moved to a shaker in a Percival chamber (22°C, continuous fluorescent light). On day 11, the media was changed to fresh liquid MS. On day 13, gentamicin was added to a final concentration of 70 mg/L. On day 15, media was decanted and seedlings were washed with 300 mL of ddH20. Chloroplasts were isolated from seedlings exactly as described above, and 3 x 10^8 chloroplasts from each line were lysed. The lysis protocol was the same as above and dot blots were also performed as above, except that lysates were diluted 1:30 before spotting.

β-Glucuronidase (GUS) Activity Assay. Three independent transgenic lines were selected for GUS analysis. Seeds from selfed primary transformants (Basta resistant) were plated on MS media without antibiotics and removed after 7 and 14 days of growth for histochemical GUS staining. Plant tissue was incubated in X-GLUC reaction buffer (2mM X-GLUC (5-bromo-4-chloro-3-indolyl b-D-glucuronide cyclohexylamine salt), 1 mM EDTA, 50 mM $NaPO_4$, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 1% Triton X-100) overnight at 37°C. Tissue was then cleared by a series of ethanol extractions before examination on a dissecting microscope.

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